



REFERENCE ONLY

UNIVERSITY OF LONDON THESIS

Degree **PhD**

Year **2005**

Name of Author **PROCTOR, J.**

COPYRIGHT

This is a thesis accepted for a Higher Degree of the University of London. It is an unpublished typescript and the copyright is held by the author. All persons consulting the thesis must read and abide by the Copyright Declaration below.

COPYRIGHT DECLARATION

I recognise that the copyright of the above-described thesis rests with the author and that no quotation from it or information derived from it may be published without the prior written consent of the author.

LOANS

Theses may not be lent to individuals, but the Senate House Library may lend a copy to approved libraries within the United Kingdom, for consultation solely on the premises of those libraries. Application should be made to: Inter-Library Loans, Senate House Library, Senate House, Malet Street, London WC1E 7HU.

REPRODUCTION

University of London theses may not be reproduced without explicit written permission from the Senate House Library. Enquiries should be addressed to the Theses Section of the Library. Regulations concerning reproduction vary according to the date of acceptance of the thesis and are listed below as guidelines.

- A. Before 1962. Permission granted only upon the prior written consent of the author. (The Senate House Library will provide addresses where possible).
- B. 1962 - 1974. In many cases the author has agreed to permit copying upon completion of a Copyright Declaration.
- C. 1975 - 1988. Most theses may be copied upon completion of a Copyright Declaration.
- D. 1989 onwards. Most theses may be copied.

This thesis comes within category D.



This copy has been deposited in the Library of UCL



This copy has been deposited in the Senate House Library, Senate House, Malet Street, London WC1E 7HU.

PROTEOMICS INVESTIGATION OF THE EFFECTS OF ENDOTHELIN-1 ON GENE EXPRESSION AND PROTEIN PHOSPHORYLATION

Jelena Predic

A thesis submitted to the University of London for the degree of
Doctor of Philosophy

August 2005

University College London



UMI Number: U593121

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U593121

Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

To Luka

ABSTRACT

This thesis describes the investigation of the effect of endothelin-1 (ET-1) on gene expression and protein phosphorylation states in two different fibroblast cell lines using proteomics tools.

Protein synthesis was monitored during the first 4 hours following stimulation of human lung fibroblasts with ET-1 using pulsed ^{35}S methionine labelling. Out of the total proteome visible on 2D gel electrophoresis 22 proteins that are differentially expressed were identified using MALDI-TOF peptide fingerprinting methodology. Changes in protein expression were temporally distinct and proteins could be classified into several different groups according to their similar kinetic behaviour possibly implying functional correlations between them.

The presence of ET receptors in NRK-49F (normal rat kidney fibroblast) cells was confirmed by calcium imaging and radioactive labelled specific ET ligand binding studies.

Changes in the phosphorylation states of proteins as well as the kinetics of these changes were assessed in NRK-49F cells following brief stimulation with ET-1. For these studies the enriched phosphoproteome was used. Enrichment was achieved by an immobilized metal affinity chromatography methodology developed in our laboratory. Individual proteins were detected with immunostaining or mass spectrometry. ET-1 stimulation was associated with phosphorylation of multiple proteins including: the members of ERK1/2 and p38 MAPK pathways and small GTP-ase pathway; an enzyme regulating calcium levels; a transcription factor; and a number of cytoskeletal,

chaperone and metabolic proteins. Nearly all of the identified proteins were represented in multiple phosphorylated forms that individually showed different kinetic behaviour during the examined period of time. The multiplicity of phosphorylations in the identified proteins has an impact on their function. The results widen the current knowledge about ET-1 signalling in NRK fibroblasts and provide a framework for further studies.

From the list of the detected phosphoproteins the phosphorylation of RhoGDI was analyzed further. By using SILAC methodology and mass spectrometry a RhoGDI peptide phosphorylated after ET-1 stimulation was detected and phosphorylation sites were mapped. This finding gives an insight into the role RhoGDI phosphorylation may have in regulating the activity of some small GTPases as downstream effectors of ET-1 signalling.

CONTENTS

ABSTRACT	3
CONTENTS	5
LIST OF FIGURES	9
LIST OF TABLES	11
ABBREVIATIONS	12
ACKNOWLEDGEMENTS	16
POSTERS AND PUBLICATIONS	17
CHAPTER 1. INTRODUCTION	18
1.1 Background	18
1.2 Endothelins	19
1.2.1 ET and tissue/cell distribution	19
1.2.2 Functional characteristics of ET	20
1.3 ET receptors	22
1.4 Physiological effects of ET	24
1.4.1 On the tissue/organ level	24
1.4.2 On the cellular level	26
1.5 Signal transduction pathways of ET receptors	28
1.5.1 G proteins	28
1.5.2 Calcium signalling	30
1.5.3 PKC signalling	31
1.5.4 MAPK signalling	32
1.5.5 Tyr kinase signalling	35
1.5.6 Other signalling pathways	38
1.5.7 Termination of signalling	38
1.6 Unexplored issues of ET–signalling and how proteomics can help	39
1.6.1 Unexplored issues	39
1.6.2 How can proteomics help?	41
1.7 Aims of Study	44
CHAPTER 2. MATERIALS AND METHODS	46
2.1 Materials	46
2.2 Cell Culture	46
2.2.1 Culture of human lung fibroblast CCD19Lu	48
2.2.2 Culture of NRK-49F	49

2.2.2.1	Freezing of NRK-49F cells	49
2.3	Pulse labelling with [³⁵S] methionine	50
2.4	Stimulation of CCD19Lu with ET-1	51
2.5	Electrophoresis of proteins	52
2.5.1	One-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE)	52
2.5.2	Two-dimensional-polyacrylamide gel electrophoresis (2D PAGE)	53
2.5.2.1	1st dimension: Isoelectric focusing (IEF)	53
2.5.2.2	2nd dimension: SDS-PAGE	54
2.6	Silver staining of polyacrylamide gels	55
2.7	Autoradiography	55
2.8	Image analysis	56
2.9	In-gel tryptic protein digestion	56
2.10	Mass spectrometric analysis	56
2.10.1	MALDI-TOF mass spectrometry	57
2.10.2	ESI-Ion trap mass spectrometry	58
2.11	ET-1 binding studies	59
2.11.1	Preparation of membranes for binding studies	59
2.11.2	Binding of ¹²⁵ I-ET-1 and competition with specific receptor inhibitors	60
2.12	Measurement of Ca²⁺ level with FLIPR (Fluorometric Imaging Plate Reader)	61
2.12.1	Preparation of cells	61
2.12.2	Calcium flux assay	61
2.13	ET-1 stimulation of NRK-49F cells	62
2.14	IMAC purification of phosphoproteins	63
2.15	Protein precipitations	64
2.15.1	TCA precipitation	64
2.15.2	Acetone precipitation	65
2.16	Protein assays	65
2.16.1	Bicinchoninic acid (BCA) protein assay	65
2.16.2	Modified Bradford protein assay compatible with IEF buffer	66
2.17	Western Blot	67
2.17.1	Chemiluminescence detection	68
2.17.2	Alkaline phosphatase detection	68
2.17.3	Stripping and reprobing membranes	69
2.18	Immunoprecipitation of RhoGDI	69
2.19	Stable isotope labelling by amino acids in cell culture (SILAC)	70
2.19.1	Cell culture media preparation and cell culture conditions	70
2.19.2	Mixing experiments	71
CHAPTER 3. PROTEOMICS MAPPING OF ET-1 INDUCED GENE EXPRESSION CHANGES IN HUMAN LUNG FIBROBLASTS		72
3.1	Introduction	72

3.2	Results	74
3.3	Discussion	89
3.4	Conclusions	96

CHAPTER 4. CHARACTERIZATION OF ET RECEPTORS IN NRK-49F CELLS

97

4.1	Introduction	97
4.2	Results	98
4.2.1	Binding studies for testing the expression of ET receptors	98
4.2.2	Characterization of functional activity of ET receptors – signal transduction	103
4.2.2.1	Ca ²⁺ flux studies	103
4.2.2.2	Activation of MAP kinase cascades in NRK-49F cells induced by ET-1	106
4.3	Discussion	111
4.3.1	Binding studies for testing the expression of ET receptors	111
4.3.2	Testing the response of NRK cells to ET-1 stimulation	113
4.3.2.1	Ca ²⁺ flux studies	113
4.3.2.2	Effects of ET-1 on MAP kinase cascades in NRK-49F cells	115
4.4	Conclusions	117

CHAPTER 5. ET-1 INDUCED PHOSPHORYLATION CHANGES IN THE PROTEOME OF NRK-49F CELLS

118

5.1	Introduction	118
5.2	Results	120
5.2.1	Two-dimensional phosphoproteome maps of NRK-49F cells	120
5.2.2	Phosphorylation kinetics	129
5.3	Discussion	135
5.3.1	Cytoskeletal proteins	136
5.3.2	Folding accessory proteins	139
5.3.3	Enzymes	143
5.3.4	Regulatory enzymes/proteins	144
5.4	Conclusions	149

CHAPTER 6. ET-1 INDUCED CHANGES OF RHOGDI PHOSPHORYLATION STATES

150

6.1	Introduction	150
6.2	Results	152
6.2.1	Immunoprecipitation of RhoGDI	152
6.2.2	MALDI-TOF analysis of immunoprecipitated RhoGDI	155
6.3	Discussion	176
6.4	Conclusions	187

CHAPTER 7. SUMMARY AND FUTURE DIRECTIONS

188

7.1	Summary	188
------------	----------------	------------

7.2	Future studies	192
7.2.1	RhoGDI	192
7.2.2	MAPK signalling	193
7.2.3	Proteomics	194
APPENDIX		196
A.	Proteins identified with MALDI-TOF peptide fingerprinting from Table 3-1	196
B.	Proteins identified with MALDI-TOF peptide fingerprinting from Table 5-1	202
C.	MALDI-TOF mass spectra	214
REFERENCES		218

LIST OF FIGURES

Figure 1-1	<i>ET isotypes and their biosynthesis</i>	20
Figure 1-2	<i>A simplified diagram of possible signalling pathways mediated by ET-1</i>	29
Figure 1-3	<i>A simplified diagram of MAPK module</i>	33
Figure 1-4	<i>General strategy for proteomic analysis of signal transduction pathways</i>	42
Figure 3-1	<i>Schematic representation of the ET-1 time course experiment with ³⁵S-Met labelling</i>	74
Figure 3-2	<i>2D map of proteins from human lung fibroblasts</i>	75
Figure 3-3	<i>2D maps of newly synthesised proteins after ET-1 stimulation of human lung fibroblasts</i>	78
Figure 3-4	<i>ET-1 induced changes in new protein synthesis are temporally dependent</i>	83
Figure 4-1	<i>Binding of ¹²⁵I-ET-1 to RMVEC</i>	100
Figure 4-2	<i>Binding of ¹²⁵I-ET-1 to NRK-49F cells</i>	101
Figure 4-3	<i>Binding of ¹²⁵I-ET-1 to HeLa cells</i>	102
Figure 4-4	<i>Ca²⁺ response in NRK cells to the application of ET-1</i>	104
Figure 4-5	<i>Effect of ET-1 on the phosphorylation of p42/p44 MAPK</i>	108
Figure 4-6	<i>Effect of ET-1 on the phosphorylation of p38MAPK</i>	109
Figure 4-7	<i>Effect of ET-1 on the phosphorylation of Raf</i>	110
Figure 5-1	<i>Quantitative changes in the total phosphoproteome of NRK-49F cells after stimulation with ET-1</i>	122
Figure 5-2	<i>Quantitative changes of protein phosphorylation in NRK-49F stimulated with ET-1</i>	123
Figure 5-3	<i>Quantitative changes of protein phosphorylation in NRK-49F stimulated with ET-1</i>	124
Figure 5-4	<i>ET-1 modifies protein phosphorylation in NRK-49F cells in a time dependent manner</i>	132
Figure 6-1	<i>Cartoon showing the role of RhoGDI in the RhoGTPase activation cycle</i>	151
Figure 6-2	<i>Immunodetection of RhoGDI after ET-1 stimulation</i>	153
Figure 6-3	<i>ET-1 induced actin polymerization</i>	154
Figure 6-4	<i>A diagram of the SILAC method applied in this chapter</i>	156
Figure 6-5	<i>2D PAGE separation of RhoGDI IP</i>	158
Figure 6-6	<i>RhoGDI mass spectrum</i>	159
Figure 6-7	<i>Sequence analysis of ion 801⁺² by MS/MS analysis</i>	165
Figure 6-8	<i>Sequence analysis of the ion 804⁺² by MS/MS analysis</i>	168
Figure 6-9	<i>The spectrum obtained from MS/MS analysis of the peak m/z = 892⁺²</i>	170

Figure 6-10	<i>Sequence analysis of the ion 892⁺² by MS/MS analysis.</i>	173
Figure 6-11	<i>Comparison of rat and mouse RhoGDI</i>	175
Figure 6-12	<i>Differences in RhoGDI isoelectric isoforms</i>	175
Figure 6-13	<i>Evolutionary conservation of IDKTDYMGSGYPRAE motif in RhoGDI proteins</i>	184
Figure 6-14	<i>Structure of the RhoGDI in complex with small GTPases</i>	185
Figure 6-15	<i>Multiple alignment of RhoGTPases</i>	186
Figure 7-1	<i>A schematic representation of the proteins and cellular processes regulated by ET</i>	190

LIST OF TABLES

Table 3-1	<i>Proteins identified by MALDI-TOF mass spectrometry</i>	80
Table 3-2	<i>Summary of identified proteins and their putative functions</i>	86
Table 5-1	<i>List of phosphoproteins from NRK-49F cells modified by ET-1 and identified by mass spectrometry</i>	126
Table 6-1	<i>Table of RhoGDI peptides recovered from spot s2</i>	160
Table 6-2	<i>Table of RhoGDI peptides recovered from spot s3</i>	161
Table 6-3	<i>Table of RhoGDI peptides recovered from spot s4</i>	163
Table 6-4	<i>Table of masses observed for the ion 801⁺²</i>	164
Table 6-5	<i>Table of masses observed for the ion 804⁺²</i>	166
Table 6-6	<i>Table of masses observed for the ion 892⁺²</i>	172

ABBREVIATIONS

2D	2 dimensional
7TM	7 transmembrane domain
ALP	Alkaline phosphatase
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BCL-2	B-cell lymphoma 2 protein
BSA	Bovine serum albumin
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CDK	Cyclin dependent kinase
cGMP	Cyclic guanosine monophosphate
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHO	Chinese hamster ovary cells
CID	Collision induced dissociation
DAG	Diacyl glycerol
DMEM	Dulbecco's modified Eagle medium
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECE	Endothelin converting enzyme
EDTA	Ethylene-diamine tetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Egr-1	Early growth response-1 gene
ER	Endoplasmic reticulum
ERK	Extracellularly regulated kinase
ESI	Electrospray ionisation
ET	Endothelin
ETR	Endothelin receptor

ET_AR	Endothelin receptor A
ET_BR	Endothelin receptor B
EST	Expressed sequence tags
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FLIPR	Fluorometric imaging plate reader
FTICR	Fourier transform ion cyclotron resonance
Gab-2	Grb2 associated protein-2
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
Grb2	Growth factor receptor binding protein-2
GRK	G-protein coupled receptor kinase
GRP	Glucose regulated protein
GTP	Guanosine triphosphate
HB-EGF	Heparin-binding EGF-like growth factor
HBSS	Hank's balanced saline solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HPLC	High performance liquid chromatography
HSP70	Heat shock protein 70
IEF	Isoelectric focussing
IF	Intermediary filaments
IL-1	Interleukine-1
IMAC	Immobilised metal affinity chromatography
IP	Immunoprecipitation
IP₃	Inositol 1,4,5-trisphosphate
IPG	Immobilised pH gradients
IRF-1	Interferon regulatory factor 1
JAK	Janus kinase
JNK	Jun N-terminal kinase
LC	Liquid chromatography
MALDI-TOF	Matrix-assisted laser desorption-ionisation time-of –flight
MAPK	Mitogen activated protein kinase
MCLK	Myosin light chain kinase

MEM	Minimal essential medium
MHC	Major histocompatibility complex
MKK	MAPK kinase kinase
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NBT	Nitro blue tetrazolium
NCBI	National centre for biotechnology information
NEAA	Non-essential amino acids
NF-kappaB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NO	Nitrous oxide
NOS	Nitric oxide synthase
NSE	Neuronal-specific enolase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PDI	Protein disulphide isomerase
PDK1	3-phosphoinositide dependent protein kinase-1
PI3K	Phosphatidylinositol-4-phosphate 3-kinase
PIP₂	Phosphatidyl-inositol 4,5-bisphosphate
PIP₃	Phosphatidyl-inositol 3,4,5-triphosphate
PKA	Protein kinase A (cAMP-dependent kinase)
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PMCA	Plasma membrane calcium transporting ATPase
PP2A	Protein phosphatase 2A
RMVEC	Renal microvascular endothelial cells
ROC	Receptor-operated channel
RT	Room temperature
S6K	Ribosomal protein S6 kinase
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulphate

Shc	Src homology 2 domain-containing protein
SILAC	Stable isotope labeling with amino acids in cell culture
siRNA	Short interfering RNA
SOC	Store-operated channels
Sos	Son of sevenless
Sox-5	SRY-box-5
SRY	Sex determining region Y
STAT	Signal transducers and activators of transcription
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TCTP	Translationally controlled tumour protein
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetate
TGF	Transforming growth factor
TNF	Tumour necrosis factor
UBF-1	Upstream binding transcription factor-1
VDCC	Voltage-dependent calcium channel
VEGF	Vascular endothelial growth factor
VIC	Vasointestinal contractor
VSMC	Vascular smooth muscle cells

ACKNOWLEDGEMENTS

Thanks are due to the following:

My primary and secondary supervisors, Prof. Jasminka Godovac-Zimmermann (Rayne Institute, UCL) and Prof. Shamshad Cockcroft (Department of Physiology, UCL) for their invaluable input and support.

For assistance with the calcium imaging study, Mr Saman Ranasinghe (Department of Physiology, UCL).

My postgraduate study was supported by a grant from Wellcome Trust, Special Trustees of the Middlesex and University College Hospitals and Proteosys AG (Mainz, Germany).

For practical help and discussions, the JGZ group past and present: Dr. Benito Canas, Chiara Guerrero, Dr Corinne Stannard, Derek Bradley, Dr. Jan Davidson-Moncada, Dr. Kajsa Paulsson, Mark Crawford, Dr. Oliver Kleiner, Dr. Regina Goetz, Sabrina Gioria, Vivien Chen and Prof. Vukic Soskic.

Prof. Anthony Segal, Dr. Jill Norman, Dr Kaveh Shakib, Dr. Ludo Dekker and numerous members of the Segal and Stewart groups (Rayne Institute, UCL).

Special thanks to: Jovan and my parents, for their patience.

POSTERS AND PUBLICATIONS

Poster presentations

- 5th Siena 2D electrophoresis meeting “From Genome to Proteome: Functional Proteomics”, 2-5th September 2002 Siena, Italy.
- SET for Britain, House of Commons, 17th March 2003, London, UK.

Publications

- Predic J., Soskic V., Bradley D. and Godovac-Zimmermann J. (2002), “Monitoring of gene expression by functional proteomics: response of human lung fibroblast cells to stimulation by endothelin-1”, *Biochemistry* 41(3):1070-1078.
- Guerrero I.C., Predic-Atkinson J., Kleiner O., Soskic V. and Godovac-Zimmermann J. (2005), “Enrichment of phosphoproteins for proteomic analysis using immobilised Fe(III)-affinity adsorption chromatography” (Accepted by *J Proteome Res*, July 2005).
- Predic-Atkinson J., Godovac-Zimmermann J. and Paulsson K.M. (2005), “Endothelin-1 induced nitric oxide release is increased and the reappearance of endothelin-1 binding sites at the plasma membrane is delayed in endothelin A receptor siRNA knock-down cells” (Submitted to *J Biol Chem*, June 2005).
- Predic-Atkinson J., Crawford M. and Godovac-Zimmermann J. (2005), “ET-1 induced phosphorylation changes in the proteome of NRK-49F cells” (In preparation).

CHAPTER 1. INTRODUCTION

1.1 Background

First reports of endothelin (ET) were noted in the late 1980s, when a new vasoconstricting agent was detected in the culture medium of human endothelial cells (Hickey *et al.*, 1985; Gillespie *et al.*, 1986; O'Brien *et al.*, 1987). Yanagisawa sequenced the vasoconstricting peptide and named it ET, later endothelin-1 (ET-1) (Yanagisawa *et al.*, 1988). With the discovery of homologous human (ET-2 and ET-3) and mouse (Vasointestinal contractor (VIC), also known as "mouse ET-2") peptides, the ET family was formed (Inoue *et al.*, 1989; Saida and Mitsui, 1991).

Until recently ET was the most powerful known endogenous vasoconstrictor (Ames *et al.* 1999). In addition ET has many other important physiological roles: in promoting development and differentiation (Kurihara *et al.*, 1994), in regulating cardiovascular and renal homeostasis (Webb *et al.*, 1998), as a neurotransmitter in the brain (Damon, 1998) and as a growth factor (Bagnato *et al.*, 1997). It has also been suggested that ET is a mediator in various diseases including: cardiovascular and renal disorders, respiratory diseases and cancer (Goldie, 1999). ET exerts its physiological effects by binding to ET receptors and thereby inducing various signalling cascades within a cell. The examples mentioned above show that ET and ET receptors have a critical role in multiple biological processes and gaining a detailed understanding of the mechanisms of their signal transduction pathways is of great importance.

1.2 Endothelins

1.2.1 *ET and tissue/cell distribution*

Endothelins, including ET-1, ET-2, ET-3 and VIC, belong to a family of disulfide bonded 21-amino acid peptides (2.5kDa) that have a multitude of physiological activities and are produced by a variety of cells and tissues (Rubanyi and Polokoff, 1994). The ET family has a strong structural resemblance (67% sequence similarity) to cardiotoxic snake venoms (sarafotoxins) which were discovered in the Israeli burrowing asp (*Atractaspis engadensis*) (Sokolovsky, 1992). This similarity indicates that endothelins and sarafotoxins may have similar biological effects.

Each ET isoform is encoded by a separate gene, and is synthesised initially as a long peptide precursor known as "pre-proendothelin" which is 200 amino acids in length (Yanagisawa *et al.*, 1988) (Fig. 1-1). These are then processed to predominantly "pro-endothelin" which is subsequently converted into ET by the activity of an endothelin-converting enzyme (ECE), a membrane bound metalloendopeptidase (Xu *et al.*, 1994).

ET-1 was first discovered in a medium of cultured endothelial cells which have proven to be the major source of ET-1 *in vivo* (Yanagisawa *et al.*, 1988). Other cell types known to produce ET-1 in humans include: vascular smooth muscle cells, epithelial cells, keratinocytes, macrophages, neurons and some cancer cell lines (Rubanyi and Polokoff, 1994).

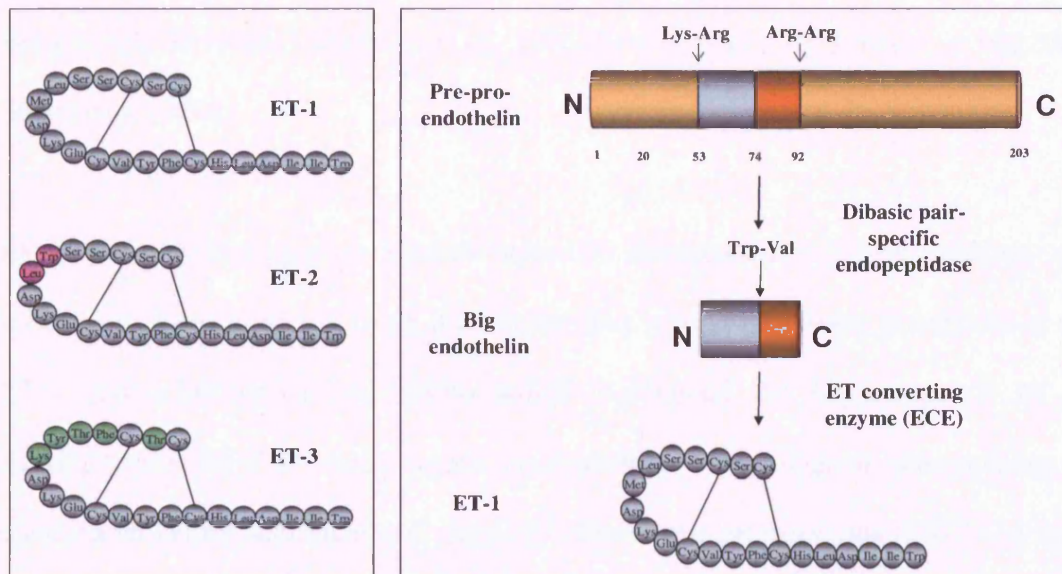


Figure 1-1 *ET isotypes and their biosynthesis.*

The expression of ET-2 and ET-3 was demonstrated in human endometrium, placenta and neurons. In addition to this ET-2 was found to be expressed in human renal adenocarcinoma (Tokito *et al.*, 1991).

The gene expression of VIC was reported in mouse intestine, uterus, ovary, stomach, lung, testis, cerebrum and cerebellum and to a small extent in kidney and heart (Uchide *et al.*, 2000).

1.2.2 Functional characteristics of ET

Once released endothelins can bind to ET-receptors either on the same cell (autocrine effect) or cells in the immediate vicinity (paracrine effect). In normal physiological conditions levels of ET in plasma are picomolar and these levels are unable to induce a biological response by activating ET-receptors (Pollock, 1998). However, in various pathophysiological states levels of circulating ET and the number of ET-receptors is

significantly elevated (Daggassan *et al.*, 1996; Sernerer *et al.*, 2000; Bruno *et al.*, 2000; Bruno *et al.*, 2002).

Experiments with transgenic animals helped to discover some of the functions that endothelins have *in vivo*. These include the first reports describing knockouts of the ET-1 gene (Kurihara *et al.*, 1994) which highlighted the importance of ET in development. ET-1 $-/-$ homozygous mice showed morphological abnormalities in craniofacial organs and increased mortality. Moreover, heterozygous (ET-1 $+/-$) mice showed a mild rise in blood pressure when compared to wild type mice. In addition, ET $-/-$ mice showed an impaired growth of the thyroid gland and thymus (Kurihara *et al.*, 1995).

Experiments with ET-2 knockouts have not yet been published, but it was mentioned at the 5th International conference on ET that these mice are apparently normal at birth but are later characterized by severe growth retardation (Webb *et al.*, 1998). Mice with targeted disruption of the ET-3 gene developed a spotted coat colour and a megacolon (Baynash *et al.*, 1994).

Levels of expression of ET isoforms vary in different stages of life (embryo, neonatal and adult) and in different tissues (Uchida *et al.*, 2002). The expression levels of ET-1 and VIC genes in mice are increased in lungs and intestines just after birth. As the lungs and intestines undergo the biggest changes of all organs functionally after birth (commencement of respiration and absorption of nutrients) the authors suggest that endothelins may play a role in the adaptation to extra-uterine life.

1.3 ET receptors

Endothelins exert their biological effects by binding to and activating ET receptors A and/or B (ET_AR and ET_BR) (Takasuka *et al.*, 1992; Sakurai *et al.*, 1992), which belong to a superfamily of G-protein coupled receptors (GPCR). A third receptor (ET_C) was found in the African horny clawed amphibious toad (*Xenopus laevis*) (Karne *et al.*, 1993).

Pharmacological studies implicated the existence of different subtypes of ET_A and ET_B receptors. Differential sensitivity to BQ-123, an ET_A receptor antagonist, enabled the subclassification of ET_A receptors into ET_A1 (BQ-123 sensitive) and ET_A2 (BQ-123 resistant) (Endoh *et al.*, 1998). Two ET_B receptor subtypes were found in rats (Gellai *et al.*, 1996). The ET_B1 localized in vascular endothelium mediated vasodilatation, whilst the ET_B2 localized in vascular smooth muscles mediated vasoconstriction.

Recent reports from Gregan *et al.* (2004 a, b) showed that ET receptors can form constitutive homo- and heterodimers. On the structural level ET receptors also show a diversity of modifications (Roos *et al.*, 1998; Stannard *et al.*, 2003 a). Stannard *et al.* (2003 a) suggested the existence of a number of receptor species that differ in the number and position of phosphorylations and palmitoylations. These examples show how ET receptor heterogeneity can affect the complexity of ET signalling.

Structurally ET receptors share similar features with other GPCRs:

- amino-terminal region located on the extracellular surface of the plasma membrane;
- 7 TM helices interconnected with 3 extracellular and 3 intracellular loops; and

- a lipid modified cytoplasmatic C terminus that forms the fourth intracellular loop.

ET_AR and ET_BR have different binding affinity for ET isopeptides (Sakurai *et al.*, 1992). ET_AR can bind ET-1 and ET-2 (ET-1>ET-2), but not ET-3 at physiological concentrations. ET_BR, on the other hand has similar binding affinity for all three isoforms of ET. This makes ET-3 the only endogenous ligand able to distinguish between ET receptor subtypes. ET_C receptor predominantly binds ET-3.

It has been shown that both ET_A and ET_B receptors are widely distributed throughout various cells and tissues in vascular endothelium, smooth muscles and also in other nonvascular structures within the heart, lung, kidney and nervous system (Rubanyi and Polokoff, 1994). ET_A receptor expression is co-localized with the expression of ET-1 and ET_B receptor expression is co-localized with the expression of ET-3 in various tissues (MacCumber *et al.*, 1989).

Gene inactivation studies of ET receptors have demonstrated that they are equally important in embryonic development as their ligands, endothelins (Clouthier *et al.*, 1998; Hosoda *et al.*, 1994). In knockout mice where both receptors were targeted only double homozygous knockouts (ET_A^{-/-} ET_B^{-/-}) showed 100% lethality (Yanagisawa *et al.*, 1998). Other knockouts displayed variable percentages of lethality, suggesting that perhaps not all ET-ETR mediated signals are essential for development.

Research of ET-ETR signalling has provided various peptide and non-peptide agonists and receptor subtype specific antagonists that have proved to be useful in delineating the mechanisms of ET receptor activation. Apart from endogenous agonists

(endothelins), sarafotoxins have also been used (S6c as ET_BR selective agonist) as well as other synthetic agonists (Davenport, 2002). Radioactively labelled endothelins are commonly used for the characterization and localization of ET binding sites. Although the major application of these reagents is in basic research, some of them are also of clinical interest (Benigni and Remuzzi, 1999).

1.4 Physiological effects of ET

The biological actions of ET can be observed on three distinct hierarchical levels which are mutually interconnected:

- the general effect of ET on tissues and organs;
- the effect on a single cell; and
- intracellular effect – signalling pathways.

1.4.1 *On the tissue/organ level*

As mentioned earlier ET has many diverse physiological effects. ET acts on different tissues and organs maintaining a balance between health and disease. As yet it is still not certain what the primary role or purpose is of such a potent and versatile biologically active substance. Its resemblance to a snake venom makes the study of ET's physiological mechanisms even more interesting.

The main role of ET appears to be in regulating the homeostasis of vascular tissues. This includes the control of blood volume, pressure, flow and viscosity (Serner *et al.*, 1995; Abassi *et al.*, 1998; Stepp *et al.*, 2001) and implicates the action of ET on diverse organ systems: cardiovascular, renal, pulmonary and others. In addition to regulating

normal organ function, it has been observed that many disorders linked to vascular systems are immediately followed by elevated levels of circulating endothelin:

- Cardiovascular disorders: acute myocardial infarction, congestive heart failure, angina pectoris, arterial hypertension, atherosclerosis (Toyo-oka *et al.*, 1991; Wei *et al.*, 1994; Hocher *et al.*, 1997);
- Diabetes: complications in diabetes (Sarman *et al.*, 1998);
- Pulmonary disorders: pulmonary hypertension, asthma (Dupuis *et al.*, 1998; Hay, 1999); and
- Renal disorders: chronic renal failure (Kohan *et al.*, 1997).

These disorders are often found to respond well to treatment with bosentan, a non-selective ET receptor antagonist (Roux *et al.*, 1999).

Another important role of ET is in development, as demonstrated by research which uses gene targeting: knockouts and mutations of ET and ET receptor genes. These experiments showed that the ET-1/ET_AR axis is essential for the development of cranial/cardiac neural crest-derived structures whilst ET-3/ET_BR signalling has a role in enteric nervous system and melanocyte development and differentiation (Kurihara *et al.*, 1994; Baynash *et al.*, 1994; Clouthier *et al.*, 1998; Hosoda *et al.*, 1994).

ET peptides and their receptors have been localized in the human brain suggesting the possibility of ET's action as a neurotransmitter (Ambar *et al.*, 1988; Naidoo *et al.*, 2004). Additionally, ET can modulate the neurotransmission in other tissues (Luciano *et al.*, 1998; Yoneyama *et al.*, 1995; Wong-Dusting *et al.*, 1989).

Angiogenesis is the process of formation of new blood vessels and in adults it occurs in wound healing and the regeneration of tissues, but it is also a critical event for the progression of tumours. In recent years research has emphasized the role of ET in angiogenesis with implications both in the processes of normal tissue vascularization and in tumour-induced neovascularization (Goligorsky *et al.*, 1999; Cruz *et al.*, 2001; Josko *et al.*, 2001; Bagnato and Spinella, 2003).

Endothelins perform other biological actions (intestinal tract movement, menstrual cycle control, release of bioactive substances) and are also associated with many different diseases (liver disorders, septic shock, rejection of transplants and cancer) (Matsumoto *et al.*, 1994; Nelson *et al.*, 1995; Watschinger *et al.*, 1996).

1.4.2 On the cellular level

All physiological effects of ET are carried out essentially on the level of a single cell, and this is then aggregated to result in a bigger tissue effect, as described in the section above. The outcome of the stimulation of cells with ET can result in a number of cell responses: cell contraction, cell growth, cell proliferation, cell transformation, cell migration and the regulation of apoptosis. Depending on the cell type and location, these effects in combination or alone give rise to tissue specific response.

ET is one of the most prominent vasoconstrictors found to date and it carries out this role by acting on vascular smooth muscles cells (VSMC). However, it has also been shown that ET is able to contract nonexcitable cells such as mesangial cells and fibroblasts (Simonson and Dunn, 1990; Fireman *et al.*, 2001; Shi-Wen *et al.*, 2004).

The proliferative function of ET was also noticed on a variety of cells and is important in ET-controlled development and differentiation, angiogenesis, wound healing, transplantation, progression of some ET related diseases and cancer (Lahav *et al.*, 1996; Alberts *et al.*, 1994; Bagnato and Spinella, 2003).

Cell proliferation together with the other cellular effects of ET all contribute to the importance of endothelins in tumour progression. (Kusuhara *et al.*, 1992; Salani *et al.*, 2000; Del Bufalo *et al.*, 2002). In addition, there is evidence for both pro- and anti-apoptotic roles of ET in different cells mediated via different receptors and intracellular mechanisms. In human melanoma cells and in rat aortic smooth muscle cells ET_B mediated the apoptotic ET-1 signal (Cattaruzza *et al.*, 2000; Okazawa *et al.*, 1998) whilst the same receptor mediated survival in rat endothelial cells (Shichiri *et al.*, 1997). On the other hand in fibroblasts ET_AR mediated anti-apoptotic effects of ET-1 (Shichiri *et al.*, 1998). In a previous study from our group proteins that regulate the cell cycle were identified as downstream targets of ET-1 in human lung fibroblasts (Stannard *et al.*, 2003 b).

Collectively, these reports show that ET uses many different ways, frequently overlapping, to perform its function. For most of the physiological effects it is still not clear what the critical step is in limiting the outcome of the ET induced cellular behaviour. The fact that there are three isotypes of ET and at least two receptor subtypes explains to a certain extent the diversity of ET induced physiological responses.

1.5 Signal transduction pathways of ET receptors

The biological effects of endothelins are initiated by the binding of these peptides to ET receptors and the subsequent activation of a cascade of cell signalling events. The network comprising endothelins, ET receptors and their signalling pathways is often termed the ET axis (Nelson *et al.*, 2003).

The ET axis can be further subdivided into the ET-1/ET_AR axis and the ET-3/ET_BR axis, because ET-3 binds only to the ET_B receptor under physiological conditions (Clouthier *et al.*, 1998). The mechanisms of signal transduction of the two subtypes of ET receptors (ET_A and ET_B) have not yet been fully documented. In the sections which follow the key components of ET intracellular signalling will be described.

1.5.1 G proteins

Commonly ET signalling pathways begin with the binding of the ligand to the heptahelical receptor. The binding of the ligand to the receptor changes the receptor's conformation and as a consequence the receptor associates with distinct classes of heterotrimeric guanine nucleotide regulatory proteins (G proteins). G proteins in their inactive form are stably associated in a heterotrimer comprising three subunits: α -subunit (that has a guanine nucleotide binding site and a GTPase activity) and β and γ subunits (that form a tight dimer) (Hamm, 1998). G proteins have been classified into four groups based on the similarity of the amino acid sequences of their α -subunits: G_s, G_{i/o}, G_{q/11} and G_{12/13} (Neer, 1995). It has been shown that ET receptors can couple to several types of G α protein subunits: G_q, G₁₁, G_s and G_i (Aramori *et al.*, 1992; Takigawa *et al.*, 1995). The exact type to which the ET receptor will couple depends on

what set of G protein subunits the particular cell expresses. After the activation of the G proteins, their α subunits exchange the bound nucleotide GDP to GTP. This reaction causes the heterotrimer to become less stable and to dissociate into an α -subunit and a $\beta\gamma$ -dimer. Both molecules can then propagate the signal further down the cascade onto a variety of effectors (Neer, 1995).

Possible signalling pathways mediated by ET are illustrated in Fig. 1-2. The possible effector molecules of $G\alpha$ and $G\beta\gamma$ in ET signalling include: PLC β , adenylate cyclase, PLD, PLA₂, guanylate cyclase and tyrosine kinases (Douglas and Ohlstein, 1997). The activation of these enzymes triggers the production or the inhibition of production of a number of second messengers such as cAMP, DAG, IP₃, cGMP, PIP₃ and phosphatidic acid.

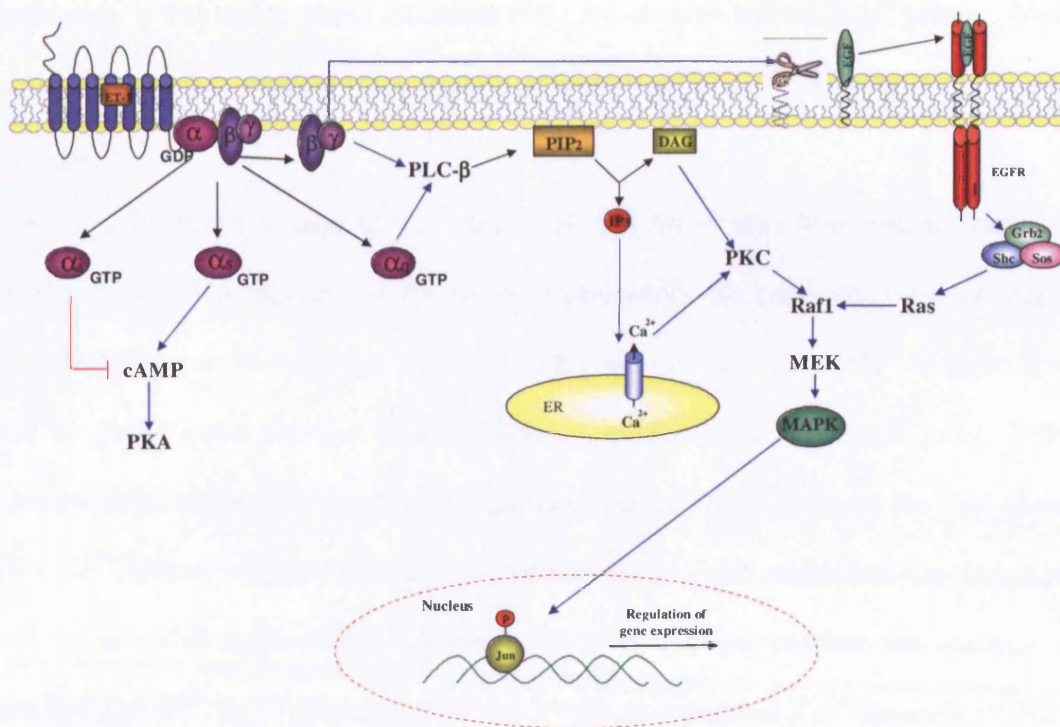


Figure 1-2 A simplified diagram of possible signalling pathways mediated by ET-1.

1.5.2 Calcium signalling

The rise of calcium (Ca^{2+}) concentration in cells stimulated with ET is a principal early response that mediates the intracellular actions of this GPCR peptide-agonist. The mechanism that elevates cytosol Ca^{2+} involves its release from Ca^{2+} -storing organelles or the influx of extracellular Ca^{2+} via a receptor-operated channel or a voltage dependent channel (Somlyo and Somlyo, 1994). In short, ET-ETR interaction triggers the $\text{G}\alpha_q$ or $\text{G}_i\beta\gamma$ activation of phospholipase C (PLC), the membrane located enzyme that mediates the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into two second messenger molecules: inositol-1,4,5 triphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Pollock *et al.*, 1995). IP_3 binds to specific Ca^{2+} sensitive IP_3 receptors in the membrane of the endoplasmic reticulum (ER) and thereby initiates Ca^{2+} release, whilst DAG is believed to mediate the activation of protein kinase C (PKC) (Fig. 1-2).

The ET-1 triggered release of Ca^{2+} from ER can result also from the activation of another type of ion channel (similar to the IP_3 receptor), the ryanodine receptor (Kai *et al.*, 1989; Wagner-Mann *et al.*, 1991). The ET evoked increase in Ca^{2+} is biphasic in that an initial rapid phase is followed by a sustained signal (Rodland *et al.*, 1991; Somlyo and Somlyo, 1994). The IP_3 induced rise in Ca^{2+} represents the first phase. This Ca^{2+} release triggers other events that can lead to cell membrane depolarization and an influx of extracellular calcium. Channels that can mediate the entrance of extracellular Ca^{2+} in ET stimulated cells are: voltage-dependent Ca^{2+} channels (VDCC) (L-type Ca^{2+} channels) (Goto *et al.*, 1989); receptor operated Ca^{2+} channels (ROC) (Iwamuro *et al.*, 1999; Simpson *et al.*, 1990; Shetty *et al.*, 1994); and store-operated channels (SOC) (Iwamuro *et al.*, 1999). Membrane ion channels other than Ca^{2+} channels may also be activated during ET-signalling namely Ca^{2+} dependent potassium

channel (K_{Ca}) (Gordienko *et al.*, 1994) and Ca^{2+} activated Cl^- channel (Takenaka *et al.*, 1992).

The rise in cytosol calcium ions makes it possible for them to bind to various Ca^{2+} -modulating proteins and propagate the signal. Probably the most well-known Ca^{2+} -binding protein is calmodulin (CaM), which regulates many different enzymes and proteins. Many proteins regulated by Ca^{2+} -CaM also take part in ET signalling including: Ca^{2+} -calmodulin-dependent protein kinases, myosin light-chain kinase (MLCK), caldesmon, calcineurin (protein phosphatase 2B (PP2B)), adenylyl cyclase, Ca^{2+} -ATPase and nitric oxide synthase (NOS) (Zhu *et al.*, 2000; Adam *et al.*, 1990; Yokomori *et al.*, 2003). Additionally, PKC, PLC, PLA_2 and PLD by themselves are Ca^{2+} -binding proteins and are important transducers of ET signalling (Schramek *et al.*, 1994; Liu *et al.*, 1992).

1.5.3 PKC signalling

The activation of PKC by ET is a well recognized event. Nevertheless, the role of this Ser/Thr kinase in ET signalling is complex. PKC is a mediator in ET-induced cell contractions as well as ET-induced mitogenesis (Pollock *et al.*, 1995; Rybin *et al.*, 1999). The mammalian PKC family of proteins can be classified into three sub-families based on sequence similarity and the type of molecule the enzymes require for their activation: the conventional PKC (cPKC), the novel PKC (nPKC) and the atypical PKC (aPKC). cPKCs (which include the isoforms: α , βI , βII and γ) are activated by phospholipid, DAG and Ca^{2+} . The nPKCs (δ , ϵ , η and θ) do not require Ca^{2+} but require phospholipids and DAG, whilst the aPKC (λ , ι , ξ and μ) are activated by phospholipids only (Toker, 1998).

ET-1 can activate the following PKC isoforms: α (Rybin *et al.*, 1999), δ (Obara *et al.*, 1999), η (Robin *et al.*, 2004; Eude *et al.*, 2000) and PKC ξ (Kanashiro *et al.*, 2000). The isoform expression is cell specific and this probably contributes to a particular cell/tissue response to ET (Ohno *et al.*, 1987). PKC is regarded as a broad specificity Ser/Thr kinase (with some differences in substrate recognition between the subclasses of PKC) that can phosphorylate Ser and Thr in the vicinity of Arg in the consensus sequence RxxS/TxxRx (a common sequence in many proteins) (Nishikawa *et al.*, 1997.). Despite the broad specificity of PKC isoforms, it seems that they have specialized roles in the cell because in addition to specific cell/tissue distribution, PKC isoforms seem to localize differentially in cellular compartments upon stimulation (Goodnight *et al.*, 1995).

1.5.4 MAPK signalling

ET can also activate gene expression and cell proliferation by adopting the mitogen activated protein kinase (MAPK) signalling pathway (Simonson *et al.*, 1992; Cazaubon *et al.*, 1993). This pathway was generally linked to the receptor tyrosine kinase signalling but recent accumulating data have revealed the involvement of G protein-coupled receptors in mitogenic signalling (van Biesen *et al.*, 1996; Gutkind, 1998). It has been well documented that ET-1 has a role as a comitogen, requiring low concentrations of some other growth stimulating factors to produce a full mitogenic response (Yang *et al.*, 1999; Hafizi *et al.*, 1999).

MAPK pathways typically follow a track of phosphorylation events (phosphorelay) during which three kinases are sequentially activated (Fig. 1-3): the extracellular signal first reaches the MKK kinase (MAPK kinase kinase) which then transfers the signal to

the MKK (MAPK kinase) by virtue of phosphorylating Ser/Thr residues (Johnson and Lapadat, 2002). Activated MKKs also have dual specificity whereby they catalyse the phosphorylation of Thr and Tyr residues on specific MAPK. MAPKs are a family of kinases that also phosphorylate various substrates including: other protein kinases, transcription factors, phospholipases and cytoskeletal proteins.

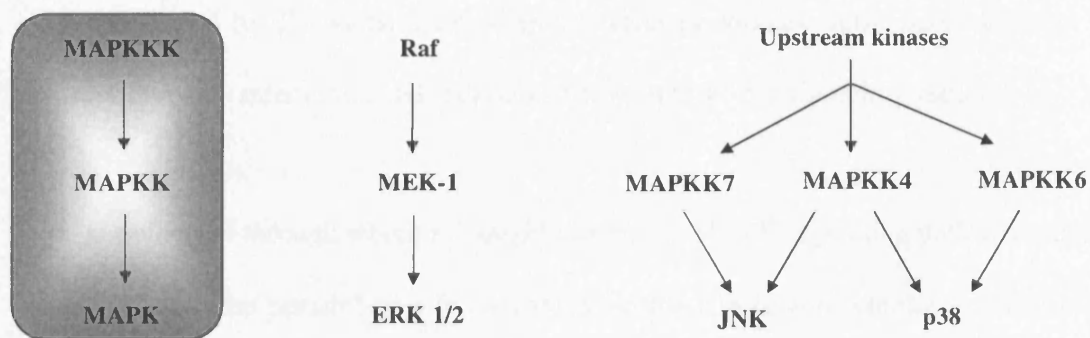


Figure 1-3 *A simplified diagram of MAPK module.*

Five members of the MAPK family have been described in mammals: extracellular signal-regulated kinase (ERK1/2), the c-Jun NH₂-terminal kinases (JNK), p38MAPK, ERK3/4 and ERK5 (Widmann *et al.*, 1999). The last two subgroups are not well characterized. These groups of MAPKs can be functionally separated by the diverse signals to which they respond and therefore activate distinct patterns of gene expression. The ERK group is involved in the signals prompting cells to respond through growth and differentiation (Gudermann *et al.*, 2000). The JNKs are activated by environmental stress signals and have a role in the regulation of cell survival and apoptosis (Touriner *et al.*, 2000). p38MAPK is also a stress activated protein kinase and has been shown to participate in inflammation, cell growth, differentiation, cell cycle and apoptosis (Ono and Han, 2000).

It was first believed that ET activates predominantly the ERK pathway, using both Gi and Gq proteins (Hilal-Dandan *et al.*, 1997; Abdel-Latif *et al.*, 2000). Recent data showed that ET could also induce the JNK and the p38MAPK pathway (Bogoyevitch *et al.*, 1995; Clerk *et al.*, 1998). The signal following the activation of GPCRs to MAPKs is delineated for ERK and JNK but not for p38 MAPK. ET-1 could therefore stimulate the pathways mediated by all three MAPKs even in the same cell suggesting that the signal mediated by ET is far more complex than previously anticipated and that it possibly involves interaction and crosstalks between different signalling routes.

Several pathways through which ET might join in the MAPK signalling pathway can be hypothesised. One possibility is the activation of the Ras protein via the $\beta\gamma$ subunit of the Gi protein which has been shown to be involved in some GPCR-mediated nuclear signalling like the Angiotensin II (Yamazaki *et al.*, 1999). On the other hand, the Gq protein can couple to the MAPK pathways both independently and dependently of PKC (Caverzasio *et al.*, 2000). Since Gq is most frequently associated with ET-1 stimulation of MAPK signalling, it is very likely that this is the route of choice that ET undertakes in most cell types where it induces proliferative effects.

Additionally, ET-1 could switch to the ERK pathway by two other mechanisms: the recruitment of β -arrestin to the ET_A receptor and the transactivation of EGFR (Imamura *et al.*, 2001; Daub *et al.*, 1996). ET receptors are internalised within 20 mins of their stimulation (Cramer *et al.*, 1997). In the process of preparation for internalisation the receptor is initially desensitised by phosphorylation (see section 1.5.7 Termination of signalling). The G $\beta\gamma$ subunit associated with the receptor mobilizes GRKs (G protein-coupled receptor kinases) which phosphorylate the ligand occupied receptor thus enabling the binding of arrestins to the receptor. Arrestins can act as docking sites for

the protein kinase Src which in turn can activate a pathway leading to ERK. It is still not clear how the signal from Src continues, but one possibility is that it phosphorylates the Shc adaptor protein, which then binds to the Grb2/Sos complex with the signal then proceeding in the usual way (Luttrell *et al.*, 1999; Hall *et al.*, 1999) (Fig. 1-2).

Another relatively recent discovery is that some GPCRs are able to induce a release of a growth-factor homologue from the membrane that binds to its corresponding growth-factor receptor and activates it. This process is termed transactivation. ET-1 was shown to transactivate EGFR by releasing an EGF-like growth factor, heparin-binding EGF (HB-EGF) (Daub *et al.*, 1996). It is thought that GPCRs activate a membrane metalloproteinase that cleaves the membrane-bound proHB-EGF, releasing HB-EGF that binds to EGFR and activates the EGF signalling pathway (Prenzel *et al.*, 1999) (Fig 1-2).

1.5.5 *Tyr kinase signalling*

ET-1 stimulation of cells increases the tyrosine phosphorylation of a number of proteins (Force *et al.*, 1991; Zachary *et al.*, 1992). The tyrosine kinases reported to be involved in ET signalling are Src, focal adhesion kinase (FAK) and the Janus kinases (JAK), all of which may play an important role in ET's mitogenic activity.

Src is a nonreceptor tyrosine kinase, activated by ET-1 (Miyamoto *et al.*, 2003). The activation of Src requires the presence of a tyrosine phosphatase to dephosphorylate Tyr⁵²⁷, which acts as an inhibitory modification by binding to and masking the kinase domain of Src (Cooper and Howel, 1993). The identity of the phosphatase allowing Src activation is still not known. Upon Src activation there are several domains through

which Src can establish its interactions with other proteins: SH2 domain binds Tyr-phosphorylated proteins, SH3 domain binds proline-rich motifs and the phosphorylated Tyr residues on Src itself can associate with other SH2-containing proteins (Cohen *et al.*, 1995). This allows Src to form multiprotein complexes accommodating various signalling proteins.

FAK is tyrosine phosphorylated upon ET-1 stimulation (Zachary *et al.*, 1992). Accumulating data suggest that FAK is the substrate of Src, as they both associate with one another and are both co-localized in focal adhesions (Calalb *et al.*, 1995; Polte and Hanks, 1997; Shen and Guan, 2001). ET-1 stimulation leads to FAK-Src complex formation (Salazar and Rozengurt, 1999). This complex can then serve as a docking site for Shc, which when phosphorylated binds growth factor receptor binding protein-2 (Grb2) and Son of sevenless (Sos). Finally, the formed complex can initiate the Ras-ERK pathway (Schlaepfer *et al.*, 1999). ET-1 also stimulates the tyrosine phosphorylation of paxillin, a cytoskeletal protein and substrate of FAK which is found in complex with FAK in focal adhesions (Zachary *et al.*, 1993).

Src and FAK are important tyrosine kinases in the processes of cytoskeletal reorganizations and the formation of actin stress fibres and focal adhesions although their precise role is not clear. These events are often triggered by growth factors and are mediated by Rho GTPase (Ridley and Hall, 1992). Focal adhesions are the sites at the cell surface where the cell cytoskeleton forms an attachment to the extracellular matrix (Petit and Thiery, 2000). They form at the sites of plasma membrane where bundles of actin stress fibres terminate. Focal adhesions modulate cell shape and motility and play a role in anchorage dependent cell growth (Ilic *et al.*, 1995; Frisch *et al.*, 1996; Schlaepfer *et al.*, 1999).

The JAK/STAT (Signal transducers and activators of transcription) pathway, first discovered in interferon regulation of gene expression, can also be activated by Gq-coupled receptors (Angiotensin II) (Marrero *et al.*, 1995). JAK tyrosine kinases associate with the activated receptor and then cross-phosphorylate one another which switches them to an active form. They can then phosphorylate STATs. Additionally they can phosphorylate the receptor itself enabling in this way the binding of different SH2-bearing signalling molecules (Taniguchi *et al.*, 1995). Phosphorylated STATs dimerize and translocate to the nucleus where they regulate gene transcription. The specificity of JAK/STAT gene activation is controlled by the type of STAT recruited to the phosphorylated receptor.

The role this pathway may have in ET signalling is unclear. Reports to date suggest that ET-1 attenuates the interleukine-induced STAT activation (Booz *et al.*, 2002), but also that ET-1 through ET_A receptor activates the STAT pathway in transfected chinese hamster ovary (CHO) cells (Peeler *et al.*, 1996). As this field is rather unexplored and there are indications that other vasoactive peptides induce STAT signalling, it is possible that the growing list of ET targets will include members of the JAK/STAT pathway in certain cells.

1.5.6 Other signalling pathways

Recent investigations by Kawanabe *et al.* demonstrated that ET-1 induces the activation of phosphoinositide 3-kinase (PI3K), with the purpose of regulating Ca^{2+} -channels (Kawanabe *et al.*, 2002; Kawanabe *et al.*, 2003; Kawanabe *et al.*, 2004). Another group showed that ET-1 stimulated nitric oxide synthase (eNOS) via the PI3K-dependent pathway (Herrera and Garvin, 2004). These reports highlight that the PI3K pathway may also be one of the mechanisms of ET-signalling but the exact role of this is not yet known. The PI3-kinases phosphorylate the hydroxyl group of the phosphatidylinositol lipids.

Protein phosphatases together with kinases are integral components of cell signalling. The activity of phosphatases can either function as a negative regulation of signalling, by rendering proteins inactive, or they can activate certain proteins by dephosphorylating their resting-cell phosphorylated form. Undoubtedly protein phosphatases play a major role in ET signalling. Examples are the phosphatases (unidentified) responsible for the activation of Src and full activation of c-Jun (Chackalaparampil *et al.*, 1994; Boyle *et al.*, 1991). Additionally ET-1 activates protein phosphatase 2B, also known as calcineurin, which has Ca^{2+} -CaM as a regulatory subunit (Zhu *et al.*, 2000).

1.5.7 Termination of signalling

GPCR mediated signals are terminated by receptor desensitisation. A negative feedback signal, mediated by a $\text{G}\beta\gamma$ subunit, mobilizes receptor kinases (GRKs) that phosphorylate the C-terminal domain of the receptor. Phosphorylation of the receptor

allows the binding of arrestins which prepare receptors for removal by endocytosis (Goodman *et al.*, 1996). Receptor endocytosis can happen via two mechanisms: clathrin coated pits and/or caveolae. Both processes of receptor internalization were reported for ET receptors (Okamoto *et al.*, 2000; Bremnes *et al.*, 2000). Deactivated receptors can be either degraded (ET_BR) or recycled (ET_AR) (Bremnes *et al.*, 2000).

1.6 Unexplored issues of ET–signalling and how proteomics can help

1.6.1 Unexplored issues

The concept of one receptor for each signalling pathway has for some time been shown to be flawed. Instead it has been shown that pathways interconnect with the pathways from other receptors forming a network of interacting proteins allowing a diverse control over cellular signalling (Daub *et al.*, 1996). In this network some proteins transfer the signals themselves by phosphorylation, dephosphorylation and hydrolysis. Other proteins have no catalytic function but instead function as adaptor proteins to bring together components that are supposed to interact with each other. This theory of cell signalling where molecules are not dynamically moving around the cell, but rather form molecular assemblies through which signals are propagated is relatively new (Hur and Kim, 2002). In this way new members of proteins such as “scaffold” and “anchor” proteins, are introduced into the “club” of cell signalling members. In addition to activating the classical second messenger cascade GPCRs can also activate the MAPK cascade and transactivate another tyrosine kinase receptor, thereby inducing a novel signalling pathway (van Biesen *et al.*, 1996; Prenzel *et al.*, 1999;). With the emerging knowledge about GPCR oligomerization, it will be interesting to discover the effects this has on the cellular signalling mediated by such receptors. Yet another characteristic

of GPCRs is that they do not couple solely to G proteins, but can interact with other types of molecules implying even greater complexity in their signalling (Hall *et al.*, 1999).

Despite the 15 year awareness of the existence of ET, the knowledge of its biological activities is still poorly understood when compared to the knowledge of other growth factors. The reason for this perhaps lies in the diversity of ET peptides and their receptors and the heterogeneity of tissue responses and signalling pathways that endothelins activate. Although a number of components of major signalling cascades triggered by ET-1 (discussed earlier) have been established, there are still a lot of underlying issues that need to be explored, for example: what are the exact mechanisms of Raf activation in certain cell types? What other transcription factors are activated by ET-1? Which phosphatase is crucial for the activation of Src and what are the downstream targets of Src leading to the mitogenic response? What is the role of FAK and Src in focal adhesion formation by ET-1 and how is this signal transduced to the nucleus? Is ET-1 an inhibitor or activator of JAK/STAT pathway, and in what cells? What are the exact roles of the activation of SAPKs and p38 MAPK and what are their upstream signalling molecules? What is the significance of the activation of all three major MAPK pathways in a certain cell (ERK, SAPK and p38)? Does ET-1 activate PI3K pathway in all cells and if so what are the components of the pathway and what is its role? How and why do certain pathways crosstalk and what is the key output signal that they determine? The number of questions seems endless and is further complicated by the ever-expanding list of proteins and possible crosstalks activated by ET in different cells.

1.6.2 How can proteomics help?

Most methods commonly used in the study of signalling pathways are methods generally applied in protein biochemistry. These include immunochemistry, chromatography, molecular biology, electrophysiology techniques as well as many others. Proteomics makes use of these classical methods but takes the study of cellular signalling one step further by employing 2D PAGE and/or various types of mass spectrometry (Godovac-Zimmermann and Brown, 2001 and 2003). 2D PAGE is a technique for the separation of proteins based on their molecular weights and their charge. The resultant protein map represents a high-resolution display of a few thousand cell proteins. These proteins can then be qualitatively and quantitatively analysed by the differential display of 2D maps from distinct cell conditions (for example control vs stimulation or health vs disease) and can be identified by mass spectrometry (MS). Additionally, posttranslational modifications of proteins can be further identified by the application of electrospray ionisation mass spectrometry (ESI-MS) which allows the sequencing of peptides by MS/MS analysis (tandem mass spectrometry) (Fig. 1-4).

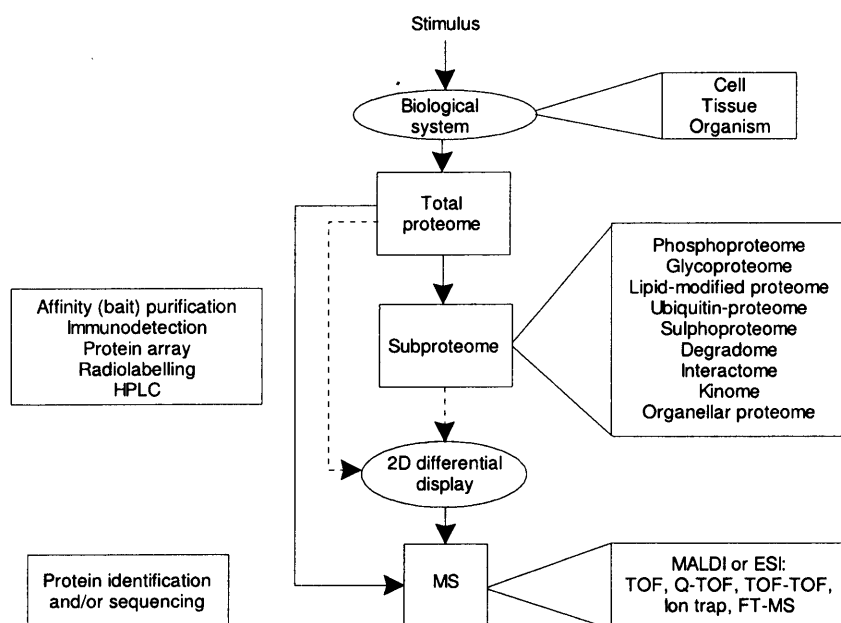


Figure 1-4 *General strategy for proteomic analysis of signal transduction pathways.*

The application of proteomics in elucidating signalling pathways has advanced in recent years and many groups focus their research on developing new proteomics-compatible techniques to help enhance the features of proteins interesting for signalling (Blagoev *et al.*, 2003; Ibarrola *et al.*, 2004). There are a number of papers describing fruitful applications of proteomics in the study of signal transduction pathways. For example 2D separation accompanied by phosphoprotein detection revealed proteins phosphorylated on Ser and Tyr upon PDGF stimulation (Soskic *et al.*, 1999 b). Kanamoto *et al.* (2002) identified 38 proteins that changed their expression level in response to TGF β treatment in Mv1Lu cells. Amongst them the down-regulation of RAD51, a new downstream target of TGF β , was demonstrated to influence genome instability. Using proteomics Pandey *et al.* (2002) identified a novel protein that was found to negatively regulate the EGFR-induced mitogenic signal. Other groups focused on the proteomic analysis of specialized cellular compartments, such as lipid rafts,

centrosomes or splicesomes, and revealed a set of new associated proteins providing evidence for additional roles of these cellular protein-organizing centres (Andersen *et al.*, 2003; Foster *et al.*, 2003; Zhou *et al.*, 2002).

Proteomics can not answer all the questions posed for the ET signalling pathway and like any other method has its limitations. These vary from simple ones like the extraction and solubility of very hydrophobic proteins to much more complex problems e.g. precise (absolute) quantification, sensitivity (in detection of proteins and MS characterization), MS identification of protein isoforms and the resolution of proteins (reviewed in Godovac-Zimmermann *et al.*, 2005). The more complex problems become especially important when applying proteomics analysis to higher eukaryotes where a large number of proteins obtained from a relatively small number of genes form diverse cells. The mechanisms that higher eukaryotes use to regulate the complex function of a cell include alternative splicing, post-translational modifications and partitioning of one protein between different isoforms. The diversity and complexity of these mechanisms places an impetus on proteomics to improve the existing and to develop new technologies capable of dealing with proteome of higher eukaryotes.

In addition to the above-mentioned limitations another weakness of current proteomics methods is the lack of quick and simple ways of analysing proteins as whole molecules as opposed to peptides obtained from protein digestion. If this problem were overcome, such analyses would enable unambiguous characterization of protein isoforms. This in turn would contribute to the understanding of functional processes with which specific isoforms are associated. In order to apply proteomics methods to signal transduction of ET we have developed new methods and we pushed to the limit currently used methods.

Two major areas that proteomics can address in ET biology are: the detection and identification of new targets downstream of the activated receptor and the qualitative and quantitative changes in ET signalling components (expression level, presence, position and extent of phosphorylation and/or other modifications). Furthermore proteomics could potentially answer even more specific questions, such as what is the phosphatase involved in Src activation.

1.7 Aims of Study

The aim of this thesis was to investigate the effect of ET-1 on gene expression and protein phosphorylation states in the cell using a proteomics based approach. In a wider context the results of these studies will provide a better understanding of the complex relationship between ET modulated proteins and consequently the events that regulate the response of a cell to ET-1.

More specifically the aims were:

1. To follow the qualitative and quantitative changes in gene expression in fibroblasts treated with ET-1 by global parallel proteomics analysis of *de novo* protein synthesis. The results of these experiments would provide an insight into which intracellular proteins are regulated by ET-1 on the level of gene transcription and consequently what are the possible cellular responses that are modulated.
2. To assess the functional activity of fibroblast ET receptors by monitoring the ET-1 induced calcium signal and the phosphorylation of MAP kinases.
3. To undertake a qualitative and quantitative global proteomics analysis of rapid changes in protein phosphorylation induced by brief stimulation with ET-1 with

a view to identifying new targets downstream of the ET receptors and the nature of their behaviour after exposure to ET.

4. To investigate the phosphorylation states of RhoGDI in resting and ET-stimulated cells and to determine its phosphorylation sites using mass spectrometry.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

Human lung fibroblasts CCD19Lu and FBS were taken from the European Cell Cultures. Normal Rat Kidney Fibroblasts (NRK-49F) and HeLa cells were kindly provided by Dr Jill Normann (Department of Medicine, Royal Free Campus, UCL). Rat renal microvascular endothelial cells (RMVEC) were a gift from Prof. Michael Goligorsky (New York Medical College, Valhalla, NY, USA). ET-1 was from Calbiochem (Nottingham, U.K.). Trypsin-EDTA, antibiotic/antimycotic, minimal essential medium, HBSS, Dulbecco's modified Eagle medium nutrient mixture F12 Ham, molecular weight marker, nonessential amino acids (NEAA) and L-Glutamine were from Invitrogen (Paisley, U.K.). Immobiline DryStrips, L-[³⁵S]methionine and Pharmalyte were from Amersham Pharmacia (Little Chalfont, U.K.). Duracryl (30% acrylamide, 0.8% bis acrylamide) was from Genomic Solutions (Huntingdon, U.K.). Complete mini-protease inhibitor cocktail was from Roche Diagnostics Ltd. (Lewes, U.K.). Sequencing grade trypsin was from Promega (Southampton, U.K.). Prestained SDS-PAGE standards low range and acrylamide were from Bio-Rad Laboratories Ltd (Hemel Hempstead, U.K.). Ni-NTA agarose was from Qiagen Ltd. (Crawley, U.K.). All other chemicals were purchased from Sigma-Aldrich Company Ltd. (Poole, U.K.) unless otherwise stated and were of the best grade available.

2.2 Cell Culture

Two fibroblast cell lines were used during the course of this thesis: CCD19Lu and normal rat kidney fibroblasts clone 49 (NRK-49F). CCD19Lu was used for the gene

expression studies. This is a human lung fibroblast cell line developed from a normal lung tissue. NRK-49F was used throughout the rest of the project due to failure of supply of CCD19Lu and their freezing incompetence. NRK-49F were derived from a mixed population of rat kidney and fibroblast cells. These cells are well characterized, they exhibit density-dependent growth inhibition and are used for growth factor bioassays.

RMVEC and HeLa cells were used for ET-1 binding experiments. HeLa cells were grown in Minimal Essential Medium (MEM) (Invitrogen Ltd., Paisley, U.K.) containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (100x solution containing 10,000U/ml penicillin-G, 10mg/ml streptomycin and 25µg/ml amphotericin-B; Sigma). RMVEC were grown in DME Ham's F12 medium containing 10% FBS and 1% antibiotic-antimycotic. Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. RMVEC and HeLa cells were cultured in 10cm culture dishes (Triple Red; Thame, Oxfordshire, U.K.) and passaged at confluence using trypsin-EDTA (Invitrogen). Stocks of cells were frozen in 90% FBS, 10% dimethyl sulfoxide (DMSO; Sigma) and stored in liquid nitrogen (BOC; Surrey, U.K.).

2.2.1 Culture of human lung fibroblast CCD19Lu

CCD19Lu cells were acquired as frozen ampoules at passage 8, from ECACC. The cell culture medium was prepared as follows:

Reagents	Final concentration
L-Glutamine	2mM
NEAA	1%
FBS	10%
Antibiotic/antimycotic mixture	0.80%
MEM with Earle's salts without L-Glutamine	500ml

Cells were grown in 10ml of medium in a water-saturated, 5% CO₂ atmosphere at 37°C in p100 polystyrene cell-culture dishes.

Cells were split 1:4 when they were 80-90% confluent. Before splitting they were washed twice with PBS. 5ml of trypsin/EDTA was added to the dish and gently agitated. The trypsin was then aspirated, and the dishes were left in the incubator at 37°C for 2 mins. Afterwards they were checked under a microscope to ensure complete detachment from the dishes. They were then resuspended in a small volume of medium and centrifuged at 4°C for 5 mins at 1000rpm. After centrifuging the washing medium was aspirated and the cell pellet was resuspended in an appropriate volume of fresh medium and plated.

Attempts were made to freeze CCD19Lu, using freezing media containing glycerol, DMSO, in FBS containing 10% DMSO and in preconditioned media taken from their own culture. However, all attempts were unsuccessful, and we were informed by the suppliers that they were unlikely to be successful under any feasible circumstances, so

the cells were continually cultured until they reached the end of their lifespan. The cells were generally able to undergo 10 splittings before showing signs of ill-health, when they were destroyed, and a new vial was started.

2.2.2 Culture of NRK-49F

NRK-49F cell line was grown in either p100 dishes or 75cm² flasks in the following medium:

Reagents	Final concentration
DMEM Nutirent Mixture F12 Ham	500ml
Antibiotic/antimycotic	1%
FBS	10%

Cells were split when they were 70-80% confluent. Splitting involved washing the cells with 10ml PBS, trypsinizing them with 2ml of Trypsin-EDTA and leaving them to incubate at 37°C for 2 mins. Detachment of cells from the dishes could be seen by the naked eye. After detaching, the cells were resuspended in fresh medium and plated.

2.2.2.1 Freezing of NRK-49F cells

NRK-49F cells were grown until 70-80% confluent. They were trypsinized as described above. After trypsinization, the detached cells were resuspended in a small volume of medium and spun for 5 mins at 1000rpm at 4°C. Supernatant was aspirated and the cell pellet was resuspended in freezing solution: 10% DMSO in FBS. The cells were first left for 1-2 days at –80°C, and then transferred to a liquid nitrogen container.

2.3 Pulse labelling with [^{35}S] methionine

Nearly confluent (80-90%) CCD19Lu cells were washed twice with a prewarmed labelling medium.

Reagents	Final concentration
MEM (Eagle) without L-Glutamine and L-Methionine	500ml
FBS	10%
Antibiotic/antimycotic mixture	0.80%
L-Glutamine	2mM
NEAA	1%

After washing, cells were incubated for 30 mins in 5ml of labelling medium in a humidified incubator at 37°C, 5% CO₂. [^{35}S] methionine was added to each dish to a final concentration of 30mCi/ml.

2.4 Stimulation of CCD19Lu with ET-1

CCD19Lu cells were incubated with or without ET-1 (final concentration 100nM) for various periods of time at 37°C. [³⁵S] methionine was added to each dish 30 mins before the cells were harvested. After the induction time the cells were washed twice with 5ml of ice-cold PBS supplemented with the following:

Reagents	Final concentration
Na ₂ P ₂ O ₇ (sodium pyrophosphate)	15mM
NaF (sodium fluoroide)	100mM
Na ₃ VO ₄ (sodium orthovanadate)	100mM
Leupeptin	2mg/ml
Trypsin inhibitor soybean	2mg/ml
Trypsin inhibitor limebean	2mg/ml
Antipain	2mg/ml

After washing cells were lysed in 270µl of isoelectric focusing (IEF) buffer:

Reagents	Final concentration
Urea	8M
Thiourea	2M
Chaps	4%
DTT	65mM
Tris base	10mM
Ampholyte	0.80%
Triton X-100	1%

Cells were scraped and sonicated (3 times with 15 second bursts at half-power at 1 minute intervals). After sonication cells were spun at 4°C for 5 mins at 13,000rpm. The pellet was discarded and the supernatant frozen at -80°C.

2.5 Electrophoresis of proteins

2.5.1 One-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

This electrophoresis was based on the protocol originally proposed by Laemmli (1970). Acrylamide used for all gels was a mixture of 30% Acrylamide and 0.8% Bisacrylamide. Separating gels were prepared in the range from 10% - 12% depending on the size of proteins. The separating gel was prepared with different volumes of 30% Acrylamide and 0.8% Bisacrylamide, then 0.375M Tris-HCl pH 8.8 and 0.1% SDS were added with APS and TEMED being used for polymerization. All stacking gels were of 5% acrylamide concentration and contained 0.125M Tris-HCl pH6.8, 0.1% SDS. The sizes of gels were either 15cm x 16cm (Hoefer SE600 system, Amersham Pharmacia) or 8.3cm x 7.3cm (Mini-Protean 3 system, Bio-Rad). Samples were prepared in SDS-PAGE sample buffer (see table below for preparation), vortexed and heated for 5 mins at 95°C.

5 x SDS-PAGE sample buffer:

Reagents	Final concentration
Tris-HCl pH 6.8	0.225M
glycerol	50%
SDS	5%
bromophenol blue	0.05%
DTT	0.25M

Gels were run submerged in electrophoresis running buffer (0.198M glycine, 25mM Tris Base, 0.1% SDS) at a constant voltage of 100V (mini gels) or 200V (large gels). They were subsequently stained by either Coomassie R250 or by silver.

2.5.2 Two-dimensional-polyacrylamide gel electrophoresis (2D PAGE)

2.5.2.1 1st dimension: Isoelectric focusing (IEF)

For the first dimension in 2D PAGE, 13 cm isoelectrofocusing strips (IPG) pH range 3-10 or 4-7 were used. 150-200µg of proteins in IEF buffer were loaded on a single strip. The rehydration step was included in the programme for isoelectric focusing. Strips were covered with mineral oil to prevent the sample from evaporating.

The following IEF programme was used:

Step	Voltage	Volt-hours(Vh)	Gradient type
1	30	330	Step-n-hold
2	3000	1500	Step-n-hold
3	4000	4000	Step-n-hold
4	6000	60000	Step-n-hold

After focusing, strips were either frozen (-20°C) or prepared for the second dimension, SDS-PAGE.

2.5.2.2 2nd dimension: SDS-PAGE

IPG strips were rinsed thoroughly with distilled water, and the excess water was drained onto a piece of filter paper. After this the strips were first treated for 20 mins with 2% DTT in equilibration buffer (30% glycerol v/v, 2% SDS w/v, 6M Urea, 0.5mM Tris-HCl, pH 6.8). The excess DTT was drained onto filter paper and IPG strips were treated with 4.5% Iodoacetamide in equilibration buffer for another 20 mins. The strips were then briefly washed with electrophoresis running buffer and were then cut at the edges (this is the area of very acidic or very basic gel where separation is very poor) and placed on top of a polyacrylamide gel. Gels (15cm x 16cm x 1mm) that were used were of 11% acrylamide concentration.

Molecular weight markers were loaded onto a piece of filter paper and placed close to the acidic part of the IPG strip onto the polyacrylamide gel. The strip and the markers were overlayed with 0.5% boiling agarose prepared in stacking gel buffer (0.125M Tris-HCl pH6.8, 0.1% SDS) with a trace of bromophenol blue. The purpose of agarose is to stop the strip from moving. The electrophoresis ran at a constant voltage of 200V for approximately 5-6 hours at 11°C. When the bromophenol blue dye reached the end of the gel the run was stopped and the gel was further stained with either silver or Coomassie brilliant blue R250.

2.6 Silver staining of polyacrylamide gels

This procedure was adopted from Shevchenko *et al.* (1996). Gels were placed in fixing solution (12% CH_3COOH and 50% methanol) and agitated for a minimum of 2 hours. They were then washed 3 times with 50 % ethanol with each wash lasting 20 mins. The gels were then pretreated for 1 min with 0.02% $\text{Na}_2\text{S}_2\text{O}_3$ and rinsed well with distilled water 3 times for 20 seconds each time. The staining step was performed in 0.1% AgNO_3 for 20 mins. Excess silver nitrate was washed off twice with water, and protein spots were detected with a solution containing 3% Na_2CO_3 , 0.0002% $\text{Na}_2\text{S}_2\text{O}_3$ and 0.009% formaldehyde. When the spots became a dark brown colour, the gels were rinsed twice for 2 mins in distilled water. Staining was stopped by immersing the gels in the fixing solution for 10 mins. After this the gels were washed in 50% methanol, and finally in 5% methanol. Gels were then packed in plastic wraps with a small amount of 5% methanol, to stop them from drying and were stored at 4°C until the proteins were cut and analysed by mass spectrometry. For autoradiography purposes gels were rinsed in 5% CH_3OH and 4% glycerol and dried between pieces of cellophane.

2.7 Autoradiography

Dried gels were exposed to autoradiography films for varying lengths of time at -80°C. At the end of the exposure the cassette was warmed to RT before being opened. The films were processed in an automated X-ray film processor (Fuji RGII).

2.8 Image analysis

Gels and autoradiography films were scanned using a UMAX Power look III scanner. Image analysis, spot matching, the analyses of changes in protein synthesis or protein phosphorylation and the estimation of pI/Mw coordinates were undertaken using Melanie III software (Geneva Bioinformatics SA).

2.9 In-gel tryptic protein digestion

The procedure is a modification of the method originally proposed by Hellman *et al.* (1995). The protein spots were cut out from the SDS-polyacrylamide gels (silver stained in most cases; for a few proteins identified in Table 3-1 a Coomassie stained gel was used) and chopped into small pieces (1mm^3). The gel pieces were treated with 25mM NH_4HCO_3 for 15 mins. This step was repeated until the pH of the solution was alkaline. No destaining of silver-stained gels was performed. Spots from Coomassie stained gels were destained in 10% CH_3OH and 10% CH_3COOH . Protein spots were then washed three times with acetonitrile and dried in the speed-vacuum concentrator for 30 mins. The dry gel clump was rehydrated in 10mM DTT and 100mM NH_4HCO_3 for 30 mins at 56°C , then spun down and the supernatant removed. Gel pieces were then treated with 100mM Iodoacetamide in 100mM Ammonium Bicarbonate for 30min at room temperature in the dark, then spun down and the liquid removed. The gel was washed again in acetonitrile and dried in the speed-vacuum concentrator. Trypsin solution was prepared by dissolving 20 μg of trypsin (sequencing grade, Promega) in 200 μl of 25mM NH_4HCO_3 . 5 μl of this solution was added to each sample, left to rehydrate on ice, and then if necessary more trypsin solution was added just to cover the gel, without letting it dry. These samples were then incubated overnight at 30°C .

For the identification of low-intensity spots (Fig. 3-2) the same spot was cut out from 2-3 silver stained gels and processed together.

2.10 Mass spectrometric analysis

2.10.1 MALDI-TOF mass spectrometry

For MALDI-TOF mass spectrometry aliquots of 0.5µl of the digest solution were applied to a target plate and allowed to air-dry. Subsequently, 0.5µl of matrix solution (1% w/v α -cyano-4-hydroxycinamic acid in 50% acetonitrile and 0.1% v/v Trifluoroacetic acid) was applied to the dried sample and the sample was again allowed to dry. Spectra were obtained using a Bruker Biflex III spectrometer (Bremen, Germany) for the gene expression studies and later a Bruker Autoflex spectrometer in reflector mode. The spectrometer was calibrated using a mixture of 7 known peptides ranging in mass between 1046 and 3495 Daltons. Obtained spectra were processed manually using Bruker's DataAnalysis software, version 1.6. Peptide spectra were internally calibrated using known autolytic peptides from trypsin. Protein fragments were identified using the MASCOT programme available at the Matrix Science web site (<http://www.matrixscience.com>) (for the identification of proteins in Table 5-1 local (intranet) Mascot version 2.1 was used), the MS-Fit programme available at the web site at the University of California at San Francisco (<http://rafael.ucsf.edu/cgi-bin/msfit>), the ProFound programme at the web site of Rockefeller University (<http://prowl.rockefeller.edu/cgi-bin/ProFound>) (for the identification of proteins in Table 3-1 the 1999/2000 version of Profound was used while proteins from Table 5-1 were identified with the Version 4.10.5) and the PeptIdent programme at the web site of EMBL in Heidelberg (<http://www.embl->

heidelberg.de/Services/PeptideSearch/FR_peptideSearchForm.html). Within search parameters 2 missed cuts, partial oxidation of methionine, carbamidomethylation and polyacrylamide adducts of cysteine were allowed. For CCD19Lu cells human database was searched. For NRK-49F cells mammalian and rodent protein databases were searched. Probability of identification was evaluated according to:

- the “P” value (probability based Mowse score, for searches in Mascot);
- the probability value, “Z” value for searches in ProFound;
- The Mowse score, for searches in MS-Fit;
- the score for searches in Peptident;
- the sequence coverage in all programmes used; and
- the reproducibility of protein identification in all peptide mass fingerprinting programmes used.

In addition to the above, the significance of a protein identification was evaluated by comparing the difference in scores between the first ranked protein and the proteins with lower ranks (second, third) in a search. The greater the difference, the higher the significance of a hit.

Even if the probability score in any single database search was not significant, identification of a protein was considered positive if the same hit was obtained in all other search programmes with highest rank, sequence coverage was high and observed Mw corresponded to the theoretical value.

2.10.2 ESI-Ion trap mass spectrometry

Tryptic digest samples were diluted 1:1 with 2% acetic acid in water. The samples were vortexed, then loaded into the micro-capillary for nanospray analysis. Nanospray mass spectrometry was performed using a Finnigan-Matt LCQ (San Jose, CA, USA), in a

positive ion mode. Collision energy changed between 20 and 40%. Peaks previously identified using MALDI-TOF MS were chosen for sequencing, both to confirm the identity of the protein, and the phosphorylation status of the tryptic peptides. Fragmentation spectra were compared to theoretical fragmentations of the likely sequences obtained from MS-Product (<http://jspl.ludwig.edu.au>), and the location of post-translational modifications was ascertained where possible. The likelihood of the modification of certain amino acids was determined using Scansite (<http://scansite.mit.edu/motifscan>) and Prosite (<http://ca.expasy.org/prosite/>).

3-2)

2.11 ET-1 binding studies

2.11.1 Preparation of membranes for binding studies

9-10 dishes of each cell line were used for membrane preparation. Cells (Renal microvascular endothelial cells (RMVEC), NRK-49F or HeLa) were grown until they were confluent. Dishes were then washed twice with 5ml ice-cold protease inhibited PBS (1 Complete mini protease inhibitor tablet /100ml of PBS). Cells were detached from the dish with 1-2ml of Trypsin/EDTA and left in the 37°C incubator for a couple of mins. Cells were then collected with 2-3ml of protease inhibited PBS and spun at 1500rpm for 5 mins at 4°C. The cell pellet was washed twice in 5ml of protease inhibited PBS. After the second wash the cell pellet was resuspended in 200µl of PBS and 2ml of ice cold H₂O and left on ice for 10 mins. Cells were harvested with a 25G needle and syringe and further lysed in a Dounce homogeniser. The suspension was spun in the Sorvall (SS34 rotor) for 60 mins at 4°C at 17,000rpm. The pellet was resuspended in 1ml of PBS and homogenised on ice using a Dounce homogeniser. An aliquot of 30µl was taken for Bicinchoninic acid (BCA) protein concentration

evaluation and the rest of the membrane suspension was frozen at -80°C or used immediately.

2.11.2 Binding of ^{125}I -ET-1 and competition with specific receptor inhibitors

All stock solutions were prepared in 0.1% BSA in PBS. For each sample a minimum of $100\mu\text{g}$ or more of membrane was used. The membranes and all other solutions were mixed together in $500\mu\text{l}$ eppendorf tubes. ^{125}I -ET-1 was the last substance added. Samples were prepared as follows to make the final volume of $200\mu\text{l}$:

Group	Membranes (μg)	^{125}I -ET-1 (pM)	ET-1 (nM)	BQ788 (nM)	BQ485 (nM)
Total binding ($\text{ET}_\text{A}\text{R}+\text{ET}_\text{B}\text{R}$)	100	200			
Non-specific (NS)	100	200	100		
NS + $\text{ET}_\text{A}\text{R}$	100	200			100
NS + $\text{ET}_\text{B}\text{R}$	100	200		100	
Total radioactivity added		200	100		

NS + $\text{ET}_\text{A}\text{R}$ and NS + $\text{ET}_\text{B}\text{R}$ were measurements when $\text{ET}_\text{A}\text{R}$ or $\text{ET}_\text{B}\text{R}$ were blocked respectively. Each group contained three to four tubes. The tubes were incubated at RT for 2 hours. They were spun at 14,000rpm for 10 mins. Supernatant was discarded and the pellet was quickly washed once with $100\mu\text{l}$ of PBS with 1% SDS. Care was taken not to disturb the pellet during washing. Afterwards the pellet was resuspended in $100\mu\text{l}$ of PBS with 1% SDS and then mixed thoroughly with 5ml of scintillation fluid. The radioactivity was measured in a γ -counter (Packard 2000 CA Tri-Carb Liquid scintillation analyzer).

2.12 Measurement of Ca^{2+} level with FLIPR (Fluorometric Imaging Plate Reader)

Measurements were conducted with the help of Mr Saman Ranasinghe (Department of Physiology, UCL).

2.12.1 Preparation of cells

2 days before the experiment, black-walled, clear-bottomed 96-well plates (Falcon) were treated with poly-L Lysine. The wells were loaded with 200 μl of 0.01% Poly-L-Lysine, and left overnight at 4°C, still, covered with parafilm. The next day liquid was aspirated from the plates and NRK-49F cells were seeded at a density of about 50,000 cells/well. The cells were plated in 100 μl of DMEM nutrient Mixture F12 Ham with 0.5% FBS. Cells were grown overnight in a water-saturated, 5% CO_2 atmosphere at 37°C.

2.12.2 Calcium flux assay

All reagents used were prepared in PBS unless otherwise stated. On the day of the experiment, media were removed, and the wells were washed twice with pre-warmed PBS. Cells were then incubated in 100 μl of fluo-4 ($C_{\text{fin}} = 2\mu\text{M}$; Molecular Probes) with probenecid (2.5mM) for 1 hour at RT in the dark. Fluo-4 stock solution was prepared in 2.5% Pluronic acid solution in DMSO. Before loading it was diluted 1:1000 in PBS. After loading the cells, the fluo-4 was aspirated, the wells were rinsed with PBS and 150 μl of PBS was added to all wells. The wells that were to show specific receptor subtype activity were preincubated with specific inhibitors for ET_A and ET_B receptor,

BQ485 (100nM) and BQ788 (100nM) respectively for 5 mins. These inhibitor solutions were then aspirated and replaced with 150 μ l of PBS and intracellular Ca^{2+} levels were subsequently assayed using the FLIPR (Molecular Devices) to simultaneously monitor fluo-4 fluorescence in all wells ($\lambda_{\text{excitation}} = 488\text{nm}$, $\lambda_{\text{emission}} = 540\text{nm}$). Cells were treated with a growing concentration of ET-1 (10nM, 100nM and 1mM). ATP (at concentrations 5 μ M, 10 μ M and 100 μ M) was used as a positive control and PBS and big-ET (1mM) as negative controls. Agonists were added to the cell plate at a velocity of 40 μ l/s. Fluorescence intensity was measured for 15 mins. Agonists were added at the time point 30 seconds, and fluorescence intensity was captured every 2 seconds for 5 mins and every 5 seconds for the last 10 mins.

2.13 ET-1 stimulation of NRK-49F cells

NRK-49F cells were grown until 50-60% confluent in DMEM Nutrient Mixture F12 Ham supplemented with 10% FBS and 0.1% antibiotic/antimycotic mixture (starvation medium). The medium was then replaced with the same medium but with 0.5% FBS. The cells were left to starve for 48h. On the day of the experiment, media were replaced with 5ml of fresh starvation medium 2 hours before the stimulation experiment. Cells were treated with ET-1 (100nM) and incubated at 37°C for various periods of time. After incubation the dishes were placed on ice, washed twice with 5ml of ice-cold PBS and lysed in 0.5ml of either immunoprecipitation-lysis buffer, or lysis buffer for IMAC phosphoprotein purification (see 2.14 and 2.15 for buffer preparation).

2.14 IMAC purification of phosphoproteins

The method described here was developed in our laboratory during the course of this thesis (see Posters and Publications). 10-20x10⁶ cells (2 p100 dishes) were used for one sample for IMAC. After stimulation the cells were lysed in the following buffer:

Reagents	Final concentration
Tris-HCl pH 7.4	50mM
Lithium dodecyl sulfate	6%
Triton-X100	6%
Na ₃ VO ₄	1mM
NaF	5mM
β-glycerophosphate	5mM
EDTA	5mM
Complete mini protease inhibitor cocktail tablet	1

The cell suspension was sonicated using sonic ultrasound (3 bursts at halfpower at 1 minute intervals) and spun for 20 mins at 13,000rpm at 4°C. LiCl was added to the supernatant to make the final concentration of 2M and the sample was vortexed briefly. One part of the bind mix (2M LiCl in lysis buffer containing 20% silica) was then mixed to 6 parts of protein solution and incubated for 5 mins on a rocking platform at RT. The suspension was centrifuged for 3 mins at 13,000 rpm and the supernatant was saved, and the silica was discarded. The protein solution was then rebuffed into binding buffer (50mM bis-(hydroxyethyl)-piperazine pH 3.4, 4% Zwittergent 3-12, 2% Triton X-100) using NAP-10 size exclusion columns (Amersham Pharmacia) according to the manufacturer's instructions. The Fe³⁺ affinity column was prepared in the following way: 1ml of 50% slurry of Ni-NTA Agarose beads was pipetted into 2.5ml plastic columns. Ni²⁺ ions were removed from the resin by washing the column with

4ml of stripping buffer (50mM Tris-HCl pH 7.5, 2% Triton X-100, 0.1M EDTA, 0.5M NaCl). The column was then washed with 4ml of water to remove any trace of EDTA and was subsequently activated with 2ml of freshly dissolved $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ (27mg/ml in water). The column was then washed with 4ml of water and equilibrated with 4ml of binding buffer. 1.5ml of protein solution containing between 1-3 mg of protein were loaded on the Fe^{3+} affinity column. At all times the column was run by gravity force alone. After loading the sample the columns were washed with 3ml of binding buffer and then with 1ml of 25mM Na_2SO_4 and 25mM imidazole in binding buffer. Phosphoproteins were eluted using 1ml of 50mM $\text{NaH}_2\text{PO}_4 \times 1\text{H}_2\text{O}$ in binding buffer. The columns, after extensive washing with binding buffer and water, can be recycled starting with the stripping buffer step.

2.15 Protein precipitations

2.15.1 TCA precipitation

Protein samples were precipitated by adding ice-cold 60% TCA to make the final concentration of 11%. In the case of IMAC elution samples, phosphoproteins were precipitated with a solution of 60% TCA and 0.2% 1,10-phenantroline. Samples were vortexed briefly and left on ice for 20 mins. They were then spun for 10 mins at 13,000 rpm at 4°C. Supernatant was aspirated and the protein pellets were washed once with ice-cold 5% TCA, and 4 times with ice-cold 80% acetone. Washing includes 5 mins incubation on ice and subsequently 5 mins spinning at 13,000rpm at 4°C. After the last wash the protein pellets were left on the bench to air-dry for 10 mins and were then resuspended in an appropriate buffer.

2.15.2 Acetone precipitation

Proteins were precipitated from solutions using 4 volumes of ice-cold acetone. Samples were left on ice for 5 mins and then spun for 5 mins at 4°C at 13,000rpm. The supernatant was discarded and the pellet was washed once with ice-cold 80% acetone. The pellet was air-dried on the bench for a couple of mins and then resuspended in an appropriate buffer.

2.16 Protein assays

2.16.1 Bicinchoninic acid (BCA) protein assay

This is a modification of a method originally proposed by Smith *et al.*, (1985). The principle of the method is that BCA reduces divalent copper ion to monovalent ion in alkaline conditions. A molybdenum/tungsten purple product is produced with an absorbance maximum at $\lambda=562\text{nm}$. Reagents used for the assay are :

- Bicinchoninic acid solution; and
- Copper (II) sulfate pentahydrate 4% solution.

A standard curve was prepared with BSA. BSA concentrations were chosen in the range between 2 μg and 100 μg , depending on the approximate estimation of the concentration of the unknown proteins.

BCA protein assays were performed in microtiter 96-well plates. The working BCA reagent solution was prepared by mixing 1 part of Copper (II) sulphate pentahydrate 4% solution to 50 parts Bicinchoninic acid solution. The working solution was added to the

BSA and unknown protein sample in the ratio of 20:1. The plate was incubated for 30 mins at 37°C. After incubation the plate was cooled to RT and absorbance measured at $\lambda=595\text{nm}$. Buffer corresponding to each set of samples was used as a control. The absorbance of the control was subtracted from the absorbance of the samples to obtain the net absorbance of the proteins. A standard curve was plotted as the net absorbance at $\lambda=595\text{nm}$ vs. the known μg of BSA. This curve was used to determine the amount of protein in unknown samples. This method is not compatible with reducing agents such as DTT.

2.16.2 Modified Bradford protein assay compatible with IEF buffer

The method was developed by Proteosys AG, Mainz, Germany and is based on methods originally proposed by Ramagli and Rodriguez (1985) and Zor and Selinger (1996).

Reagents:

- Coomassie working solution: Coomassie reagent (BioRad) was diluted 1:4 with H_2O ;
- 0.02N HCl;
- Diluted IEF buffer: IEF buffer (see section 2.4) was diluted 1:5 with 0.02 N HCl; and
- Standard protein stock solution: 10mg/ml BSA (fraction V, Sigma) in IEF buffer. Stock solution was frozen in 100 μl aliquots.

Dilution series of BSA for the standard curve were prepared by diluting the BSA stock solution with dilute IEF buffer. Unknown protein samples were diluted 1:5 with 0.02N HCl. Each protein sample was then mixed in the ratio of 1:40 with Coomassie working

solution, incubated for 5 mins at RT and the absorbance was read at $\lambda = 595\text{nm}$ and at $\lambda = 450\text{nm}$. To determine the protein concentration of the unknown samples, the ratio of $\lambda_{595}/\lambda_{450}$ was plotted against the amount of standard protein. Each experiment was carried out in triplicate.

2.17 Western Blot

Reagent solutions prepared for Western Blot are:

- Transfer buffer: 20% methanol, 0.1% SDS, 25mM Tris base and 192mM glycine;
- TBS (Tris-buffered saline): 10 mM Tris-HCl pH 7.4, 170 mM NaCl;
- TBS/Tween: 0.1% Tween 20 in TBS;
- Ponceau stain: 0.1% ponceau dye in 7% TCA; and
- Blocking buffer: 5% nonfat dry milk, 1% BSA in TBS/Tween buffer.

For Western Blots proteins were first separated on 10% SDS-PAGE gels, and then transferred onto a nitrocellulose membrane in a semi-dry blotting system. The transfer was performed at 15V for 30 mins and then at 25V for 90 mins. The transfer of the proteins was checked with ponceau staining. Membranes were blocked in blocking buffer for 2 hours at RT or overnight at 4°C. Following this the membranes were washed 4 times for 5 mins with 50ml TBS/Tween buffer and incubated with the primary antibody overnight at 4°C. The primary antibody was diluted in TBS/Tween buffer according to the manufacturer's advice. Membranes were washed 4 times with 50ml TBS/Tween for 5 mins and incubated with the secondary antibody for 1-2 hours at RT. Secondary antibodies were mostly horseradish peroxidase conjugated, and in some

cases they were alkaline phosphatase conjugated. The dilutions of secondary antibodies were 1:10,000 in TBS/Tween (for ALP conjugated or 1:4000 of TBS/Tween buffer for ALP conjugated antibodies). After incubation the membranes were washed 3 times for 5 mins with 50ml of TBS/Tween and were then washed once with 50ml of TBS.

2.17.1 Chemiluminescence detection

After washing the nitrocellulose membranes, the washing buffer was poured off and the membranes were covered with a 1:1 mixture of Amersham "ECL" detection reagents, just enough liquid to cover the blot. Membranes were incubated for 1 min and the excess liquid was drained away on a filter paper leaving the blot moist. The blot was then wrapped in saran wrap and exposed serially to film in a cassette for 1 mins, 2 mins and 5 mins. For 2D electrophoresis blots Amersham "ECL Plus" reagents were used in a ratio of 40:1. Membranes were incubated with the reagent mix for 5 mins and were then exposed to film and developed.

2.17.2 Alkaline phosphatase detection

The following stock solutions were prepared:

1. NBT: Dissolve 5g of NBT in 10ml of 70% dimethylformamide (DMF);
2. BCIP: Dissolve 0.5g of BCIP (disodium salt) in 10ml of DMF; and
3. ALP buffer: 100mM NaCl, 5mM MgCl₂, 100mM Tris (pH 9.5).

All stocks are stable at 4°C for at least 1 year.

Just prior to developing, fresh substrate solution was prepared. 66µl of NBT stock solution was added to 10ml of ALP buffer, mixed well and the 33µl of BCIP stock was added. The working solution should be used within 1 hour. This solution was added to the blots with enough being used to cover the membranes and the stain was developed at RT with agitation until it was suitably dark. Rinsing the membranes with PBS containing 20mM EDTA chelating the Mg^{2+} ions stopped the reaction.

2.17.3 Stripping and reprobing membranes

This method can be used to retest blots that were detected with the chemiluminescent method.

The following stripping buffer was prepared: 62.5mM Tris-HCl pH6.8, 10mM β -mercaptoethanol and 2% SDS TBS/Tween buffer.

Stripping buffer was pre-warmed for 5 mins at 60°C. Membranes were soaked in stripping buffer and slowly agitated for 30 mins at 60°C. After incubation the membranes were washed with plenty of TBS/Tween buffer twice for 10 mins. The membrane could then be reprobed following the Western Blot procedure starting from the step of blocking of the membranes.

2.18 Immunoprecipitation of RhoGDI

After stimulation with ET-1 (see 2.13), NRK-49F cells were washed with PBS and scraped in lysis buffer containing Tris-buffered saline with 1% Triton X-100, 1mM Na_3VO_4 , 5mM NaF, 5mM β -glycerophosphate, 5mM EDTA, 2mM $Na_2P_2O_7$ and 1

Complete Mini Inhibitor cocktail tablet. Cells were disrupted by repeated aspiration through a 21 gauge needle and sonication (3 bursts for 10 seconds each 1 min apart). Cell homogenates were transferred to a microcentrifuge and centrifuged at 13,000 rpm, 30 mins at 4°C. The protein concentration was determined from the supernatants with the BCA. Equal amounts of proteins in the supernatants were precleared with 0.1 µg rabbit IgG, 20µl of protein A-sepharose and 50µl Sepharose CL4B for 30 mins rotating at 4°C. The pellet was collected by centrifugation at 2,500 rpm for 30 seconds at 4°C and was discarded. The supernatant was transferred to a fresh microcentrifuge tube and incubated for 1h at 4°C with 2µg of anti-Rho GDI antibodies per 500µg of cell proteins. Protein A-conjugated agarose beads (Perbio) were added and incubated overnight. Immunoprecipitates were collected by centrifuging at 2,500 rpm for 5 mins at 4°C. Supernatant was discarded and pellets were washed 3 times with 500µl ice cold lysis buffer. Finally, the pellets were washed with 500µl of ice-cold 10mM Tris-HCl pH 7.4. Bound proteins were eluted with 200µl of IEF buffer without DTT for 1.5h at RT. DTT was added to the sample at a final concentration of 65mM prior to isoelectric focusing and 2D gel electrophoresis.

2.19 Stable isotope labelling by amino acids in cell culture (SILAC)

2.19.1 Cell culture media preparation and cell culture conditions

DMEM Nutrient mixture F12 Ham without arginine was used. The arginine-free medium was divided into two lots and L-arginine (Sigma) and L-arginine-U-¹³C₆ (Cambridge isotope labs) were added separately. The medium with its full complement of amino acids was sterile filtered through a 0.22µm filter (Millipore, Bedford MA) and stored at 4°C for later use.

NRK-49F cell line was grown in DMEM Nutrient mixture F12 Ham, prepared as described earlier (see 2.2.2), supplemented with 10% filtered fetal bovine serum plus antibiotics, in a humidified atmosphere with 5% CO₂ in air. Cell lines were grown for at least six cell divisions in labelling media containing either normal arginine or ¹³C6-Arg before the stimulation experiment (see 2.13).

2.19.2 Mixing experiments

For this kind of experiment in addition to the control (no stimulation) only one stimulation time point was used (4 mins with ET-1 (100nM)). After stimulation and lysis of the NRK-49F cells, protein concentrations were determined using the BCA protein assay and then lysates (control and stimulation) were combined in the ratio of 1:1 (normal Arg: ¹³C6-Arg). The mixed cell lysates were then used for RhoGDI immunoprecipitation.

CHAPTER 3. PROTEOMICS MAPPING OF ET-1 INDUCED GENE EXPRESSION CHANGES IN HUMAN LUNG FIBROBLASTS

3.1 Introduction

ET regulates cell growth and proliferation by activating signalling cascades that follow the pathway to the nucleus where they culminate in the activation of gene expression (Cazaubon *et al.*, 1993; Sakurai *et al.*, 1994; Yang *et al.*, 1999; Hafizi *et al.*, 1999). The transcription of early genes, c-jun, c-fos and c-myc, was demonstrated within 3 hours of ET-1 stimulation in fibroblasts and mesangial cells (Pribnow *et al.*, 1992; Simonson *et al.*, 1992). Neyses *et al.* (1991) showed that ET-1 also induces new proteins synthesis in rat cardiomyocytes and that this process is controlled by the transcription of the early growth response-1 (Egr-1) gene. In addition, ET's role in regulating cell cycle progression was connected with *de novo* synthesis of proteins in mouse fibroblasts, rat astrocytes and mesangial cells (Suzuki *et al.*, 1999; Pedram *et al.*, 1998; Teixeira *et al.*, 2000).

At present, changes in gene expression are usually monitored by nucleotide-based methodologies such as expression profiling (Hughes and Shoemaker, 2001). However, the nucleotide-based methods have a number of technical and conceptual disadvantages compared to direct analysis of cellular proteins: (A) the old paradigm of one gene equals one protein is not correct for eukaryotic cells where splice variants, posttranslational modifications, etc. lead to perhaps six to eight proteins per gene on average (Strohman, 1994); (B) while 2D electrophoresis methods are commonly used to resolve and display most cellular proteins (O'Farrell, 1975), analogous multidimensional display methods

are not available for polynucleotides (mRNA); (C) the amplification of different mRNAs may not be quantitatively faithful, and the correlation between mRNA levels and cellular protein levels is not high (Anderson and Seilheimer, 1997; Gygi *et al.*, 1999; Griffin *et al.*, 2002); (D) the nucleotide-based methods are not suitable for following the plethora of posttranslational modifications of proteins which are vital to cellular response and regulation.

In the recent years proteomics approaches to studying gene expression have been successfully applied. For example Kanamoto *et al.* (2002) followed changes in protein abundances by using 2D differential display of proteins in stimulated versus unstimulated systems. Another approach uses liquid chromatography coupled to Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometry to study gene expression in microorganisms (Pasa-Tolic *et al.*, 2002; Kolker *et al.*, 2005). In addition to this, metabolic labelling was used to proteomically detect expression levels of proteins in nematodes (Krijgsveld *et al.*, 2003).

The sections of this chapter which follow describe a functional proteomics approach to studying early gene expression after ET-1 stimulation of human lung fibroblasts. The experimental strategy applied uses pulsed [³⁵S] methionine labelling to detect newly synthesized proteins. For proteins which showed changes in new protein synthesis between stimulated and unstimulated cells, the identity of the protein was determined by high-sensitivity MALDI-TOF mass spectrometry.

3.2 Results

Confluent dishes of human lung fibroblasts CCD19Lu were stimulated with ET-1 (100nM) for 30, 45, 60, 120 and 240 mins respectively. All dishes were stimulated at the same time point (0 min). ^{35}S -Met ($C_{\text{fin}}=700\mu\text{Ci/dish}$) was added to the cells 30 mins before cells were collected for harvesting (Fig. 3-1). Each dish of cells had its own control being dishes of cells that were labelled with ^{35}S -Met but not stimulated with ET-1.

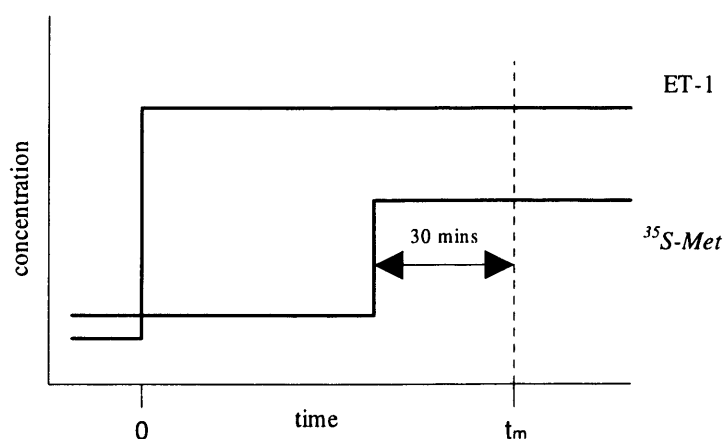


Figure 3-1 *Schematic representation of the ET-1 time course experiment with ^{35}S -Met labelling.* Cells were stimulated with ET-1 at time $t=0$. 30 mins before cells were harvested ^{35}S -Met was added to the dishes. Cells were harvested after incubation with ET-1 for a certain period of time (t_m).

Proteins from the cell extract were first separated according to their isoelectric point (pI) values on immobilized pH gradient strips (pH 4-7). After first dimension separation the proteins were separated again on SDS-PAGE according to their molecular weights and their electrophoretic mobility. The 2D protein map created from these two separation processes was stained with silver (Fig. 3-2 A).

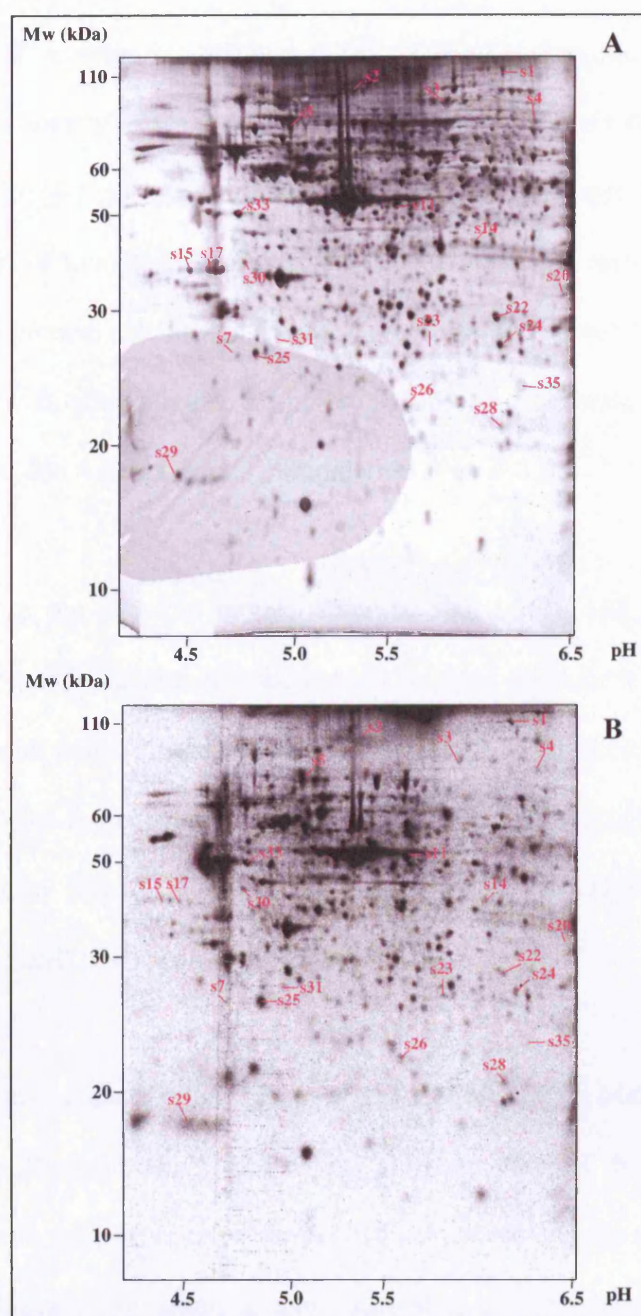


Figure 3-2 2D map of proteins from human lung fibroblasts. (A) Proteins separated by isoelectric focusing and SDS-PAGE were stained with silver. (B) New protein synthesis was detected with ^{35}S labelling of proteins. The image shows an autoradiograph of the gel image in A. The proteins that were identified with MALDI-TOF peptide fingerprinting are labelled on the gels. Spot identification numbers correspond to Table 3-1.

Many thousands of proteins are expressed in a typical eukaryotic cell, and this is reflected in the complexity of the 2D PAGE patterns observed for total protein extracts from CCD19Lu (Fig. 3-2 A). Around 2000 total proteins were reproducibly present on the 2D PAGE gels of human lung fibroblasts. In contrast, the response of cells to a stimulus may only involve new protein synthesis for a limited subset of the total cellular proteins. A much smaller number of proteins showed detectable amounts of new protein synthesis within 4 hours of ET-1 stimulation (Fig. 3-2 B).

To detect which of the proteins in the silver stained image had changed in their expression level, the 2D gels obtained were dried between cellophane paper and placed in direct contact with autoradiography film and enclosed in a light-tight cassette for 3 days at -80°C . After the exposure time, the films were photographically processed. The images obtained (Figs. 3-2 B, 3-3) show a 2D map of proteins with ^{35}S -Met incorporation as a result of *de novo* protein synthesis.

The autoradiographs obtained were scanned and analysed with Melanie III software (Genebio, Geneva Bioinformatics SA). This software enabled detection of protein spots, matching them against those on the control gels, analysing the changes in protein synthesis, quantifying these changes and estimating pI and Mw coordinates and statistical analysis. Separate analyses for each set of gels were undertaken (control gel and its corresponding stimulated gel). The proteins that showed variations in intensity between stimulated gels and control gels provided an indication of which spots were good candidates to be cut out and identified. A total of about 40 proteins showed detectable differences in protein synthesis between stimulated and unstimulated cells. For 22 of these proteins, which showed the largest changes in synthesis, their identity has been determined by MALDI-TOF mass spectrometry (Table 3-1). These spots were

then cut out of the gels and digested with trypsin. The resulting peptide-digests were analysed in a MALDI-TOF mass spectrometer. The mass spectra obtained were processed manually with the help of Bruker's data analysis software. This involved recalibrating the spectra and labelling the peaks. The peak values were then run through the search engines of several protein databases.

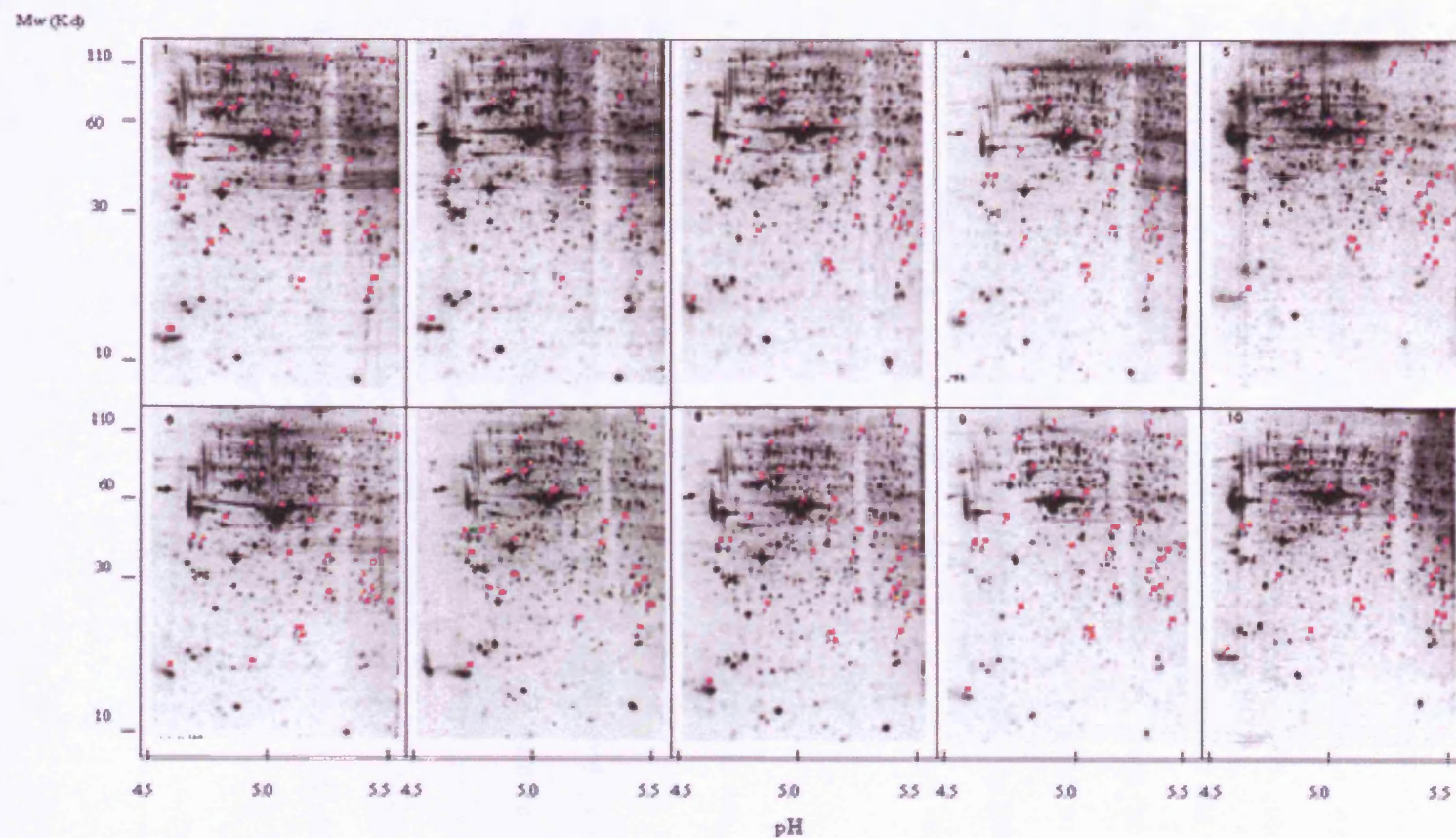


Figure 3-3 2D maps of newly synthesised proteins after ET-1 stimulation of human lung fibroblasts. Incorporation of ^{35}S -Met in proteins was monitored after cells were incubated with ET-1 (lower panel) or without ET-1 (upper panel) for 30 mins (gels 1 and 6), 45 mins (gels 2 and 7), 60 mins (gels 3 and 8), 120 mins (gels 4 and 9) and 240 mins (gels 5 and 10). Autoradiographs of each time point are presented.

For most of these proteins the mass spectrometry identifications were straightforward. Specific splice variants were observed for vinculin and nucleolar transcription factor (Table 3-1). In addition, conflicts in the sequence databases were resolved, e.g., the Thr/Ala conflict at position 4 of Ras-related protein Rab-14 (Appendix 1). A low molecular weight form of Rab-14 (Table 3-1) was observed. A particularly interesting case was the observation of two forms of Rab-14 (s23 and s28, Table 3-1) which showed very different new protein synthesis (see below). From the mass spectrometry results, the higher molecular weight form of Rab-14 appears to correspond to the complete gene sequence, whereas the lower molecular weight form appears to correspond to a splice variant with deletions at at least one end of the sequence (Appendix 1).

The changes in new protein synthesis were time dependent, and groups of proteins showing different kinetic behaviour could be observed (Fig. 3-4). Thus, for example, filamin, Sox5, and plasminogen activator inhibitor 2 all showed rapid changes in protein synthesis between 30 and 60 mins followed by a constant level of up-regulation (filamin, Sox5) or down-regulation (plasminogen activator inhibitor 2) at later times (Fig. 3-4 panel A). Other groups of proteins showed very different kinetic behaviour (Fig. 3-4 and Table 3-1). The Rab-14 (long) and Rab-14 (short) isoforms also showed very different kinetic behaviour (Fig. 3-4 panels D and E).

Spot	Gene ¹	Protein	Gi ²	Mw obs. ³	Mw	pI obs.	pI	no of peptides ⁴	% coverage ⁵	Δ mass ⁶	Score ⁷
1. Cytoskeletal proteins											
s1	VCL	Vinculin	4507877	117	115	5.8	6.1	20	40	0.12	119
s15	TPM2	Tropomyosin 2	42476296	33	35	4.6	4.5	6	19	0.15	88
s17	TPM3	Tropomyosin 3	136085	33	33	4.6	4.6	5	18	0.06	61
s29	MYL6	Myosin light polypeptide 6	17986264	17	14	4.46	4.5	6	45	0.09	50
2. Folding Accessory Proteins											
s5	HSPA5	Heat shock 70 kDa protein 5 (GRP78)	6470150	71	78	5.9	5.0	12	24	0.24	139
3. Enzymes											
s3	GANAB	Glucosidase	2274968	107	107	5.7	5.8	13	20	0.26	60
s22	CYP11A 1	Cytochrome P-450-scc	189438	28	29	7.2	6.1	6	26	0.1	52
s24	OAZ	Ornithine decarboxylase antizyme	852428	24	27	7.8	6.2	5	20	0.04	48

Table 3-1 *Proteins identified by MALDI-TOF mass spectrometry.* (Continued overleaf)

Spot	Gene ¹	Protein	GI ²	Mw obs. ³	Mw	pI obs.	pI	no of peptides ⁴	% coverage ⁵	Δ mass ⁶	Score ⁷
4. Regulatory proteins											
s2	SF3B2	Splicing factor 3B subunit 2	55749531	98	108	5.5	5.34	9	11	0.19	61
s4	UBTF	Nucleolar transcription factor 1 (UBF-1)	136652	89	91	5.6	6.3	13	19	0.11	87
s14	SOX5	SRX (sex determining region Y)- box 5	1881852	39	40	6.5	6.0	10	25	0.16	79
s23	RAB14	Ras-related protein Rab-14.	20379074	24	25	7.8	6.1	6	33	0.29	43
s28	RAB14	Ras-related protein Rab-14	6563200	24	21	5.8	6.18	6	40	0.05	62
s31	RAB3A	RAB3A, member RAS oncogene family	4506367	25	25	4.9	4.9	7	33	0.14	60
s33	RPSA	40S ribosomal protein SA; laminin- binding protein	125969	33	48	4.8	4.8	6	32	0.12	86
s35	CDKN1B	Cyclin-dependent kinase inhibitor 1B	1168871	22	22	6.5	6.3	6	32	0.21	61

Table 3-1 (cont'd) *Proteins identified by MALDI-TOF mass spectrometry.* (Continued overleaf)

Spot	Gene ¹	Protein	GI ²	Mw obs. ³	Mw	pI obs.	pI	no of peptides ⁴	% coverage ⁵	Δ mass ⁶	Score ⁷
5. Other											
s7	FLNC	Filamin C, gamma	938227	24	25	4.8	4.7	5	35	0.15	53*
s11	SERPINB2	Plasminogen activator inhibitor-2 precursor	1352712	47	47	5.5	5.5	5	12	0.05	86
s20	ANXA4	Annexin 4	1703319	36	33	5.8	6.4	5	21	0.14	43
s25	TPT1	Tumour protein, translationally-controlled 1	136479	20	24	4.8	4.8	5	21	0.04	71
s26	BCL2A1	Bcl2-related protein A1	4757840	20	21	5.3	5.6	5	24	0.21	60
s30	NPM1	Nucleophosmin	825671	31	37	4.7	4.7	4	26	0.13	40

Table 3-1 (cont'd) *Proteins identified by MALDI-TOF mass spectrometry.* (1) Name of the gene coding for the identified protein; (2) NCBI sequence identifier; (3) Mw estimated from SDS-PAGE; (4) Number of MS identified peptides in the database search; (5) Percentage of protein sequence covered by the identified peptides; (6) Average deviation between theoretical and experimental masses for each matched peptide; (7) Probability based Mowse score taken from Mascot.

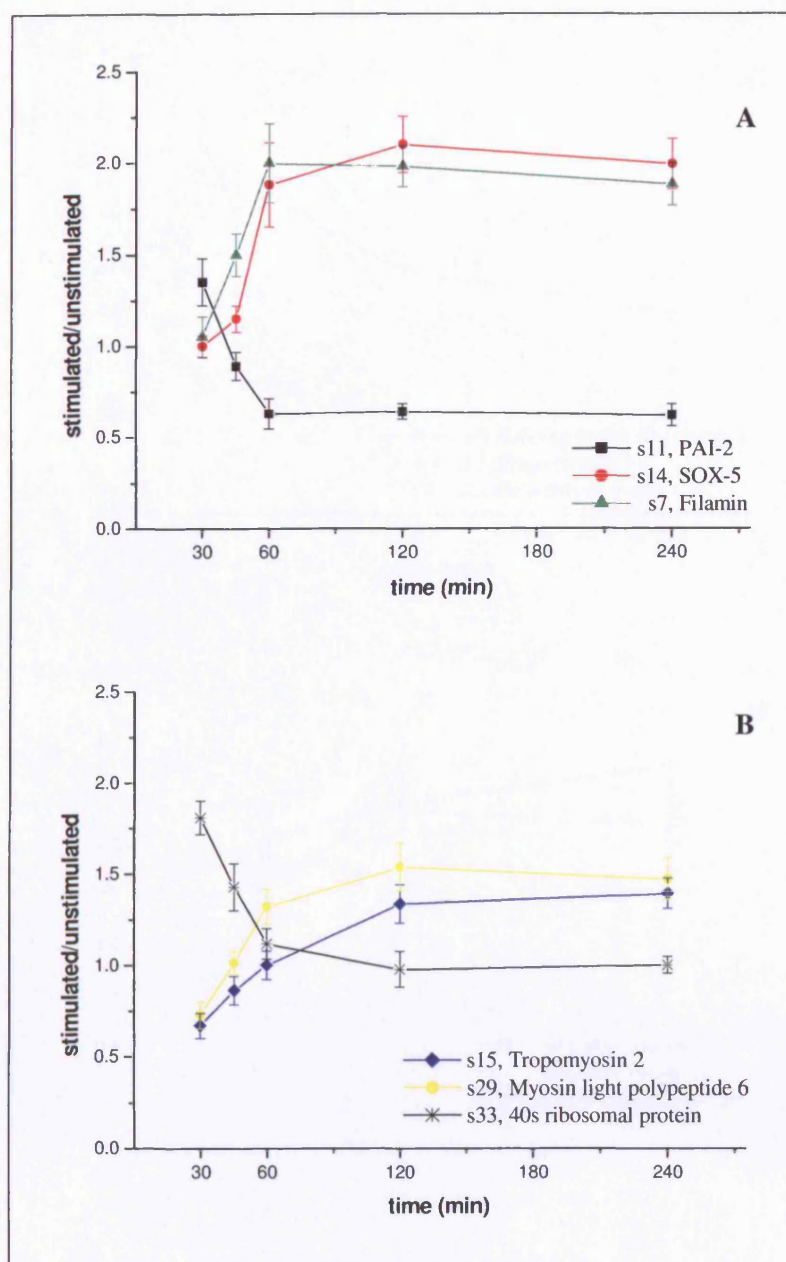


Figure 3-4 *ET-1 induced changes in new protein synthesis are temporally dependent.*
(Continued overleaf)

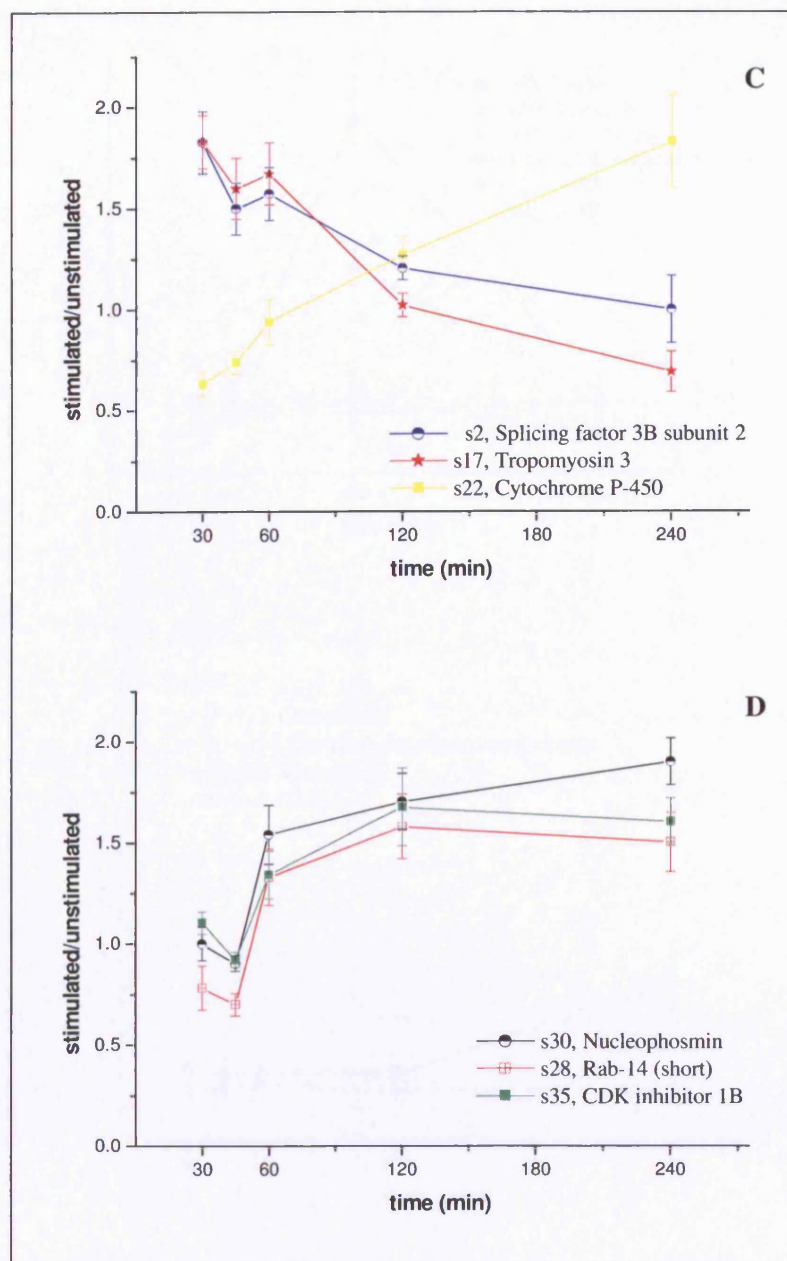


Figure 3-4 (cont'd) *ET-1 induced changes in new protein synthesis are temporally dependent.* (Continued overleaf)

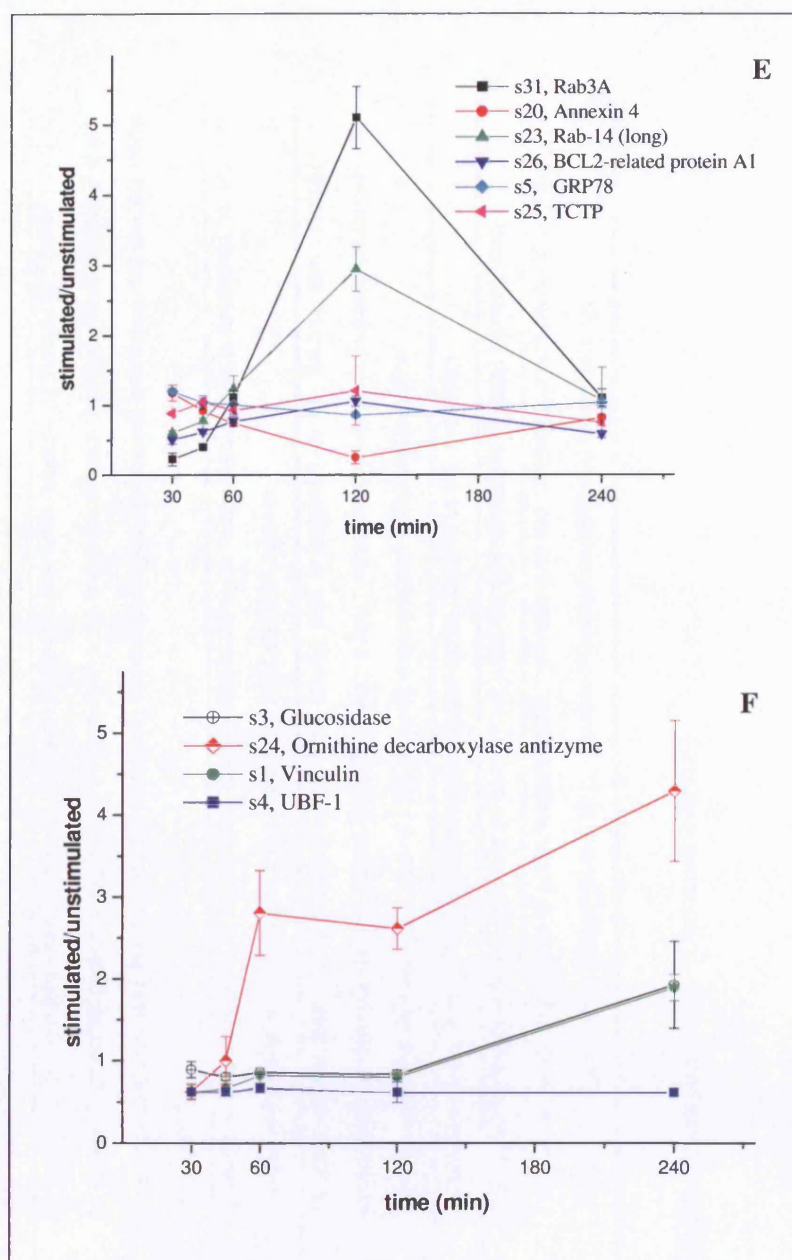


Figure 3-4 (cont'd) *ET-1 induced changes in new protein synthesis are temporally dependent.* The level of new protein synthesis is presented as the ratio of spot intensities in cells stimulated with ET-1 to spot intensities in unstimulated cells. This ratio is plotted against ET-1 incubation time. Separate panels have been used to group proteins which show similar time dependence (panels A-E). Statistical analysis was carried out by Dr Vukic Soskic (Proteosys AG, Mainz, Germany).

Functional group	Protein	Function
Cytoskeletal	Tropomyosin	Binds to actin filaments in muscle and non-muscle cells. In non-muscle cells it is implicated in stabilizing the cytoskeleton.
	Vinculin	Cytoskeletal protein associated with cell-cell and cell-matrix junctions, where it is thought to function as one of several interacting proteins involved in anchoring F-actin to the membrane. Multiple alternatively spliced transcript variants have been found for this gene, but the biological validity of some variants has not been determined.
	Myosin light chain	Myosin is a hexameric ATPase cellular motor protein. Myosin alkali light chain is expressed in smooth muscle and non-muscle tissues and is a regulatory light chain of myosin.
Chaperones	Heat shock 70 kDa protein 5	Member of the heat-shock protein-70 (HSP70) family and is involved in the folding and assembly of proteins in the endoplasmic reticulum (ER). May play a key role in monitoring protein transport through the cell. Its (over)expression is associated with reduced apoptosis and cell transformation.
Enzymes	Glucosidase	A metabolic enzyme involved in processing oligosaccharides, located in ER and Golgi.
	Cytochrome P450	Ubiquitously expressed. Large family of enzymes. Part of the pathway of cholesterol biosynthesis.
	Ornithine decarboxylase antizyme	Plays a role in the regulation of polyamine synthesis by binding to and inhibiting ornithine decarboxylase. Antizyme expression is auto-regulated by polyamine-enhanced translational frameshifting.

Table 3-2 *Summary of identified proteins and their putative functions.* (Continued overleaf)

Functional group	Protein	Function
Regulatory	Splicing factor 3B subunit 2	Together with splicing factor 3a and a 12S RNA unit, forms the U2 small nuclear ribonucleoproteins complex (U2 snRNP). Involved in mRNA processing.
	Nucleolar transcription factor 1	A transcription factor required for the expression of ribosomal RNAs.
	SRY (sex determining region Y)-box 5	A transcription factor involved in the regulation of embryonic development and in the determination of the cell fate.
	Rab-14	Belongs to small GTPase family. Important in intracellular vesicle trafficking.
	Rab 3A	Belongs to small GTPase family. Important in exocytosis, neurotransmitter secretion.
	40S ribosomal protein SA	A structural constituent of ribosomes, also known as laminin binding receptor. Has a role in cell adhesion and is downstream of S6 kinase.
	Cyclin-dependent kinase inhibitor 1B	Binds to and prevents the activation of cyclin complexes, and thus controls the cell cycle progression at G1.

Table 3-2 (cont'd) *Summary of identified proteins and their putative functions.* (Continued overleaf)

Functional group	Protein	Function
Other (unknown)	Annexins	A family of Ca^{2+} -dependent phospholipid binding proteins with various roles: in cell motility, inflammation, signal transduction, excocytotic and endocytotic pathways.
	Filamin	Promotes orthogonal branching of actin filaments and links actin filaments to membrane glycoproteins. Anchors various transmembrane proteins to the actin cytoskeleton and serves as a scaffold for a wide range of cytoplasmic signalling proteins.
	Plasminogen activator inhibitor-2	Inhibits urokinase-type plasminogen activator. Inhibits TNF-alpha induced apoptosis.
	Tumour protein, translationally-controlled 1	Found in normal and tumour cells. Unknown function.
	Bcl-2-related protein A1	A member of the BCL-2 protein family which act as anti- and pro-apoptotic regulators that are involved in a wide variety of cellular activities. This protein is up-regulated by different extracellular signals such as NF-kappa B, TNF and IL-1.
	Nucleophosmin	Ubiquitously expressed phosphoprotein involved in ribosome assembly/transport, cytoplasmic/nuclear trafficking, regulation of DNA polymerase alpha activity, centrosome duplication, and regulation of p53.

Table 3-2 (cont'd) *Summary of identified proteins and their putative functions.* Proteins whose abundance was altered by ET-1 stimulation were grouped according to their putative roles in the cell and the reported areas of functions within a cell are summarized. Information was collected from Human Genome resources at <http://www.ncbi.nlm.nih.gov/genome/guide/human/> and from Uniprot protein resource at <http://www.expasy.uniprot.org/>. See discussion for references.

3.3 Discussion

The purpose of this study was to investigate the immediate short-term response of human lung fibroblasts to stimulation with ET-1 by monitoring the level of synthesis of new proteins using proteomics tools. Newly synthesised proteins were pulse labelled with ^{35}S -methionine. Out of approximately 2000 protein spots seen on the gel, 40 spots showed a change in intensity between unstimulated and stimulated cells. Of these 22 were unambiguously identified (Figs. 3-2, 3-3 and Table 3-1).

The set of proteins which show significant changes in protein synthesis over short time periods after ET stimulation (Table 3-1) includes proteins which have been implicated in a very wide range of different cellular and extracellular processes. This diversity presumably reflects the fact that the cell's response to a mitogenic signal such as ET stimulation results in major changes in cellular function at many levels. These changes include intracellular changes connected ultimately with cell division, extracellular interactions with neighboring cells, and homeostasis at the organismal level. On the other hand, the number of proteins observed in the early response to ET stimulation is a very small subset of the total human genome, suggesting that the proteins that have been observed may have key roles in facilitating and directing this response. The observations set out in this chapter may suggest new roles or reinterpretations of the roles of individual proteins in human lung fibroblasts.

Changes in the fluxes of new protein synthesis were highly time dependent and the induction of these changes occurred on a similar time scale to signalling responses observed by phosphorylation/dephosphorylation (Godovac-Zimmermann *et al.*, 1999).

This supports the proposition that the observed changes in protein synthesis are a direct consequence of ET-1 signalling.

One of the most interesting groups of kinetically related proteins is the group shown in Figure 3-5 panel E: Rab3A, Rab-14 (long), annexin IV, Bcl2-related protein A1, translationally controlled tumour protein, and heat shock 70 kDa protein 5 (GRP78). Rab proteins are known to be important in intracellular vesicle trafficking (Takai *et al.*, 2001). Rab-14 has been observed in brain, spinal cord, heart, kidney, and lung cells, but there do not seem to be any studies of its physiological function nor any reports of variant isoforms. In contrast, Rab3A is one of the best studied of the many Rab proteins and is known to play a key regulatory role in Ca^{2+} -dependent exocytosis, particularly in neurotransmitter release from nerve terminals (Takai *et al.*, 2001).

The translationally controlled tumour proteins (TCTPs) are a highly conserved and abundantly expressed family of eukaryotic proteins. They are implicated in cell growth and the human acute allergic response but their intracellular biochemical function has remained elusive. Recently, on the basis of the solution structure of the TCTP from *Schizosaccharomyces pombe* (Thaw *et al.*, 2001), it has been suggested that the TCTPs might form a structural superfamily with the Mss4/Dss4 family of proteins. These latter proteins bind to the GDP/GTP free form of Rab proteins and have been termed “guanine nucleotide-free chaperones”. The result in this chapter is the first reported experimental observation of a possible functional linkage between Rab proteins and TCTP. Furthermore, given the role of Rab3A in Ca^{2+} -dependent exocytosis and the fact that the TCTPs are also calcium binding proteins (Kim *et al.*, 2000), it is intriguing that annexin IV is also included in this group of kinetically related proteins, albeit with changes in

new protein synthesis which are the opposite of those observed for Rab3A, Rab-14, and TCTP (Fig. 3-4 panel E).

The annexins are a family of calcium and phospholipid binding proteins whose physiological functions are poorly understood and for which many diverse functions have been proposed, including in vesicle trafficking and as intracellular ion channel regulators (Hawkins *et al.*, 2000). Furthermore, new synthesis of heat shock 70 kDa protein 5 parallels that of annexin IV, suggesting that it might be especially important for the folding of annexin IV. Finally, Bcl2-related protein A1 is a well-known antiapoptotic protein (Jäätelä, 1999).

It seems likely that this group of proteins is involved in some form of vesicular trafficking which, despite the large changes in Rab3A, seems unlikely to be an exocytotic process and might be related to the endocytosis and recycling of the ET receptor (Bremnes *et al.*, 2000). However, this group of proteins might also be involved in cytoskeletal rearrangements given recent reports that Rab proteins may be involved in microtubules (Takai *et al.*, 2001; Somsel Rodman and Wandinger-Ness, 2000) and that TCTP has been reported to bind to microtubules (Gachet *et al.*, 1999). In addition the link between heat shock 70 kDa protein 5, Bcl2-related protein A1 and apoptosis indicates that the proteins from this group might be important in cell survival (Breckenridge *et al.*, 2003).

A curious feature of the experiments presented in this chapter was that an isoform of Rab-14 with a lower M_w (s28, Table 3-1) and very different kinetic behaviour (Fig. 3-4 panel D) was also observed. The very different kinetic behaviour of the two isoforms of Rab14 presumably reflects the regulation of the response to ET at the level of mRNA

splicing. The variant form of Rab14 was grouped kinetically with nucleophosmin (Fig. 3-4 panel D), which is an abundant, multifunctional nucleolar protein (Table 3-2). Nucleophosmin has been reported to have a possible role in the assembly and/or transport of ribosomes, molecular chaperone activity and ribonuclease activity (Hingorani *et al.*, 2000). Other reports show that nucleophosmin is important in centrosome duplication via phosphorylation by cell cycle kinases (Okuda *et al.*, 2000; Tokuyama *et al.*, 2001). Cyclin-dependent kinase (CDK) inhibitor 1B followed a similar expression pattern and is included in this kinetically related group of proteins. Up-regulation of this protein negatively regulates cell proliferation (Polyak *et al.*, 1994). These results suggest that this group of proteins are not involved in ET's proliferative effects and may have other functions.

The present results also monitor a couple of proteins which are generally connected with various aspects of RNA synthesis. These include proteins involved in the transcription of rRNA (nucleolar transcription factor 1) or in RNA splicing (splicing factor 3b, subunit 2) (Table 3-2). Nucleolar transcription factor 1 (UBF-1) is one of the essential components of the initiation complex for RNA polymerase I (Hannan *et al.*, 1998). For nucleolar transcription factor 1, the following contributes to the regulation of rRNA transcription:

- the quantity of this protein (Hannan *et al.*, 1998);
- posttranslational phosphorylation by G1-specific cdk-cyclins (Voit *et al.*, 1999);
- acetylation (Hannan *et al.*, 2000); and
- interactions with the product of the retinoblastoma susceptibility gene Rb (Pelletier *et al.*, 2000).

In these experiments it was observed that the expression of UBF-1 is down-regulated by about 40% within 30 mins of ET-1 stimulation and remains suppressed (Fig. 3-4 panel F). This would seem to be consistent with reports that this protein is inactive in early G1 phase (Klein and Grummt, 1999). Very different behaviour is shown by splicing factor 3b, subunit 2. This protein is up-regulated by about 80% 30 mins after stimulation with ET-1 followed by a slow decrease to the synthesis levels observed in unstimulated cells (Fig. 3-4 panel C). It is particularly interesting that changes in synthesis were observed for single proteins that are components of large multiprotein complexes, e.g., only subunit 2 of the 4 different proteins comprising splicing factor 3b. This seemingly anomalous result might be explained if these proteins have special roles in the initiation or localization of such complexes.

40S ribosomal protein SA showed an initial up-regulation of about 80% at 30 mins followed by a slow decay to normal synthesis levels (Fig. 3-4 panel B). It is likely that this is connected to its alternative role as a laminin binding protein at the cell surface (Buto *et al.*, 1998) rather than as a component of the ribosome. The up-regulation of this protein is observed in a variety of different types of cancer (Menard *et al.*, 1998). The same protein is identified later in this thesis (see Chapter 5, Table 5-1) where it was phosphorylated upon ET-1 stimulation of rat fibroblasts. 40S ribosomal protein is phosphorylated by S6 kinase, which has been reported to be activated by ET in cardiac myocytes (Wang *et al.*, 2001).

The proteins shown in Table 3-1 also include a number of proteins involved in cytoskeletal interactions. Cytoskeletal interactions are known to be important in a wide variety of cellular processes including signalling systems, cell-cell interactions, and

mitosis. The up-regulation in some cytoskeletal proteins could suggest that ET induces cytoskeletal reorganisation (Gohla *et al.*, 1999).

One of the more unexpected results of the experiments set out in this chapter was the observation of the up-regulation of the transcription factor Sox5 (also known as SRY (sex determining region Y)-box 5) (Fig. 3-4 panel A). This protein has previously only been reported in the context of spermatogenesis with localization in the adult testis (Bowles *et al.*, 2000; Wegner, 1999). Although the Sox transcription factors have been widely studied, this has usually been in the context of cellular differentiation and embryonic development. Among the hundreds of papers on Sox transcription factors, only a few deal specifically with Sox5, and there do not seem to be any reports dealing with the involvement of Sox5 in response to a signalling process or with the role of Sox5 in fibroblasts. Neither is there any evidence suggesting why Sox5 might be functionally linked with filamin or plasminogen activator inhibitor 2 (Fig. 3-4 panel A). It can be speculated either that Sox5 may have other types of roles or that it is particularly important in maintaining the identity of the differentiated human lung fibroblasts.

Together with the kinetic groupings, which may reflect functional links, these experiments provide a framework for further investigation. A few proteins were identified for which either:

- the variant observed seems not to be present in sequence databases (short form of Rab-14); or
- the previously discussed functional roles do not give any clear relationship to ET stimulation (Sox5); or
- they do not have any known functional role at present (TCTP, Table 3-2).

All of these are candidates for further investigation.

The proteins which showed changes in new protein synthesis in the experiments presented in this chapter were different to those observed in previous studies of ET stimulation of: mouse fibroblast cells (Suzuki *et al.*, 1999); astrocytes (Pedram *et al.*, 1998; Teixeira *et al.*, 2000); and rat mesangial cells (Terada *et al.*, 1998). These earlier studies investigated ET's effects on cell cycle progression and showed strong increases of cyclins D1 and D3, cdk4 kinase, p16INK4, p21cip1, and the phosphorylated form of pRb. Changes in the expression of several protooncogenes (c-fos, c-jun, c-myc from vascular smooth muscle cells and Egr-1 from cardiomyocytes) following ET stimulation have also been reported (Reiss *et al.*, 1993; Simonson *et al.*, 1992; Yang *et al.*, 1999; Neyses *et al.*, 1991). The difference in proteins observed may be a consequence of the different cells but probably also reflects the different time scales of the experimental protocols. The previous experiments measured changes in total protein concentrations of maximally 2-5-fold following 4-24 h of constant exposure to ET (Reiss *et al.*, 1993; Yang *et al.*, 1999; Suzuki *et al.*, 1999; Pedram *et al.*, 1998; Teixeira *et al.*, 2000; Terada *et al.*, 1998). This type of experiment measures the adaptation of the cells to long-term exposure to ET and therefore may reflect generalized changes consistent with cell growth rather than new protein synthesis that is an early consequence of ET stimulation. The detection of cellular responses after short time exposures to ET is likely to be more informative about the immediate interconnectivities of cellular pathways.

Apart from the 25-fold change in the rates of new protein synthesis for Rab-14 (long) observed over the time course of the experiments, all of the other proteins showed changes of less than 5-fold and many were less than 2-fold (Fig. 3-4). This seems to be consistent with the growing number of expression profiling experiments that typically

observe limited changes in a large number of mRNAs rather than on/off behaviour for a few genes (Miklos and Maleszka, 2001).

3.4 Conclusions

- ET-1 stimulated *de novo* protein synthesis in human lung fibroblasts within 4 hours of stimulation.
- The level of protein expression depended on the length of the cell's exposure to ET-1.
- 22 proteins with the biggest patterns of change (ET-1 stimulated/unstimulated) were identified amongst which there were at least four functionally distinct groups of proteins: cytoskeletal, chaperones, regulatory proteins, enzymes and proteins with multiple or not clearly defined functions. This implies that ET stimulation results in major changes in cellular function at many levels.
- Proteins that followed similar temporal patterns of up/down- regulation after ET-1 stimulation were grouped together (Fig. 3-4). Such clustering could possibly indicate functional correlations between proteins of the same kinetic group.
- The results obtained support and further develop the idea of partitioning of functional processes downstream of ET receptors (Stannard *et al.*, 2003 a; Godovac-Zimmerman and Brown, 2003). The architecture of the signal is likely to follow the pattern of parallel activation of different pathways rather than one signalling pathway which at a certain point diverges into several different branches.

CHAPTER 4. CHARACTERIZATION OF ET RECEPTORS IN NRK-49F CELLS

4.1 Introduction

The two GPCR through which ET acts are ET_AR and ET_BR. Both ET_AR and ET_BR are widely distributed throughout various cells (Rubanyi and Polokoff, 1994). ET_AR is predominantly expressed in vascular smooth muscle cells and causes prolonged vasoconstriction (Pollock *et al.*, 1995). ET_BR is predominantly expressed in endothelial cells and causes vasodilation (de Nucci *et al.*, 1988). In addition it was recently suggested that complex interactions between the two ET receptor subtypes in renal microcirculation can modulate renal function. ET_BR stimulation produced renal vasoconstriction but when co-stimulated with ET_AR the net effect was vasodilation (Just *et al.*, 2004).

The vasoconstrictive effects of ET are mediated through the increase of intracellular calcium (Ca²⁺) levels, initially via IP₃ triggered release of Ca²⁺ from the endoplasmic reticulum and later via the influx of extracellular ions through Ca²⁺-channels (Pollock *et al.*, 1995; Iwamuro *et al.*, 1999). In addition to cell contraction, Ca²⁺ signalling is responsible for other biological effects of ET-1 (discussed in Chapter 1) and can be described as a marker of the ET induced cell signal.

ET's mitogenic effect is mediated via the activation of MAPK signalling cascades (Simmonson *et al.*, 1992; Cazaubon *et al.*, 1993). All three members of MAPKs (ERK, JNK and p38) were reported to take part in ET signalling in different cells and tissues (Cazaubon *et al.*, 1993; Clerk *et al.*, 1998; Bogoyevitch *et al.*, 1995).

The aim of the experiments presented in this chapter was to establish the suitability of NRK-49F cells for studying the signal transduction pathways of ET. NRK cells are an immortalized rat kidney cell line often applied in studies of growth factors and cell transformation (Lahaye *et al.*, 1999). In the experiments presented in this chapter radioligand binding assays were used to perform an initial screening of several cell lines (RMVEC, HeLa and NRK-49F) for the presence of ET receptors. The expression of ET_A and ET_B receptor types was evaluated using selective competitive antagonists. The functional activity of the receptors was measured by the ability of a ligand-occupied receptor to induce a Ca²⁺ response and to activate the phosphorylation of various MAP kinases in NRK-49F cells. These studies revealed that both ET receptors are present and functionally active in rat fibroblasts. ET induced an elevation of calcium and the activation of c-Raf-1 as well as the activation of both ERK1/2 and p38MAPK in NRK-49F cells. These findings provided a good model system to further study the mechanisms of ET-mediated cellular actions.

4.2 Results

4.2.1 Binding studies for testing the expression of ET receptors

Radio-labelled ligand binding experiments were performed as initial screening studies for the presence of ET receptors on three cell lines: RMVEC, NRK-49F and HeLa cells. Membranes isolated from these cells were used for competitive binding experiments to estimate the binding sites for endothelin. If a radio-labelled ligand is allowed to bind to a cell or membrane sample, the binding can be indirectly measured by measuring the radioactivity of that sample. In the first stage of the binding studies the membranes were incubated with ¹²⁵I-ET-1 (200pM) alone and the total binding of the radioactive

ligand to the cell membranes (to both receptors) was determined ($ET_A R + ET_B R$, Figs. 4-1, 4-2 and 4-3). To estimate the binding to non-specific sites (NS), the membranes were incubated with the radioactive ligand but in the presence of an excess of unlabelled ET-1 ($1\mu M$). Specific binding was determined from the difference between the total and the non-specific binding. The difference between the total and the non-specific binding in RMVEC was statistically insignificant and showed that these cells do not express ET receptors (Fig. 4-1). Specific binding in both NRK and HeLa cells was statistically significant (Figs. 4-2 and 4-3), indicating the presence of ET receptors in these cells.

To differentiate between ET receptor subtypes (ET_A and ET_B) specific competitive antagonists for ET_A and ET_B receptors were used. BQ485 was used as an $ET_A R$ antagonist with $IC_{50} = 3.4\text{ nM}$ for $ET_A R$ (Itoh *et al.*, 1993). To selectively block $ET_B R$, the BQ788 antagonist was used, with $IC_{50} = 1.2\text{ nM}$ for $ET_B R$ (Ishikawa *et al.*, 1994). Membranes were incubated with ^{125}I -ET-1 (200pM) in the presence of 100nM BQ485 (NS+ $ET_A R$) or 100nM BQ788 (NS+ $ET_B R$). Binding to a specific type of ET receptor was determined as follows:

$$ET_A R \text{ binding sites} = (ET_A R + ET_B R) - (NS + ET_B R)$$

$$ET_B R \text{ binding sites} = (ET_A R + ET_B R) - (NS + ET_A R)$$

An unpaired t-test was used to compare the data of total radioactivity binding and binding in the presence of selective receptor antagonist. Both NRK-49F and HeLa cells express both ET_A and ET_B receptors (Figs. 4-2 and 4-3). From the results obtained it appears that the $ET_B R$ type is the predominant species in NRK-49F and HeLa cells.

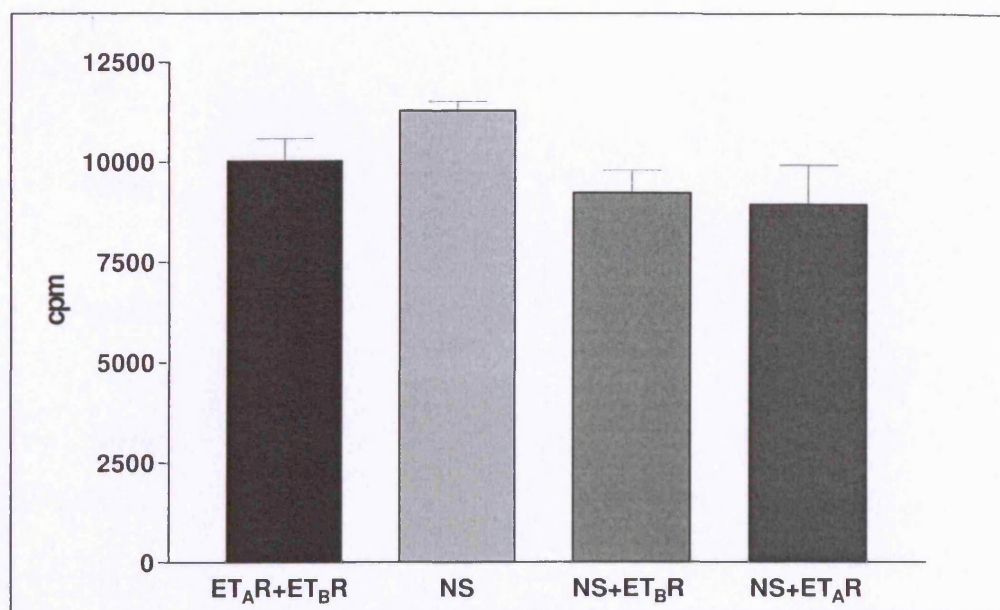


Figure 4-1 *Binding of $^{125}\text{I-ET-1}$ to RMVEC.* Membranes from RMVEC were incubated with $^{125}\text{I-ET-1}$ (200pM). Bound radioactivity was measured in the presence of: 1 μM unlabelled ET-1 (NS), 100nm BQ485 (NS+ET_BR), 100nM BQ788 (NS+ET_AR) or with no competitor (ET_AR+ET_BR). The difference between ET_AR+ET_BR and NS represents the total specific binding to both receptors. The data shown are the mean values of $n=3\pm\text{SEM}$ except for ET_AR+ET_BR group where $n=4$.

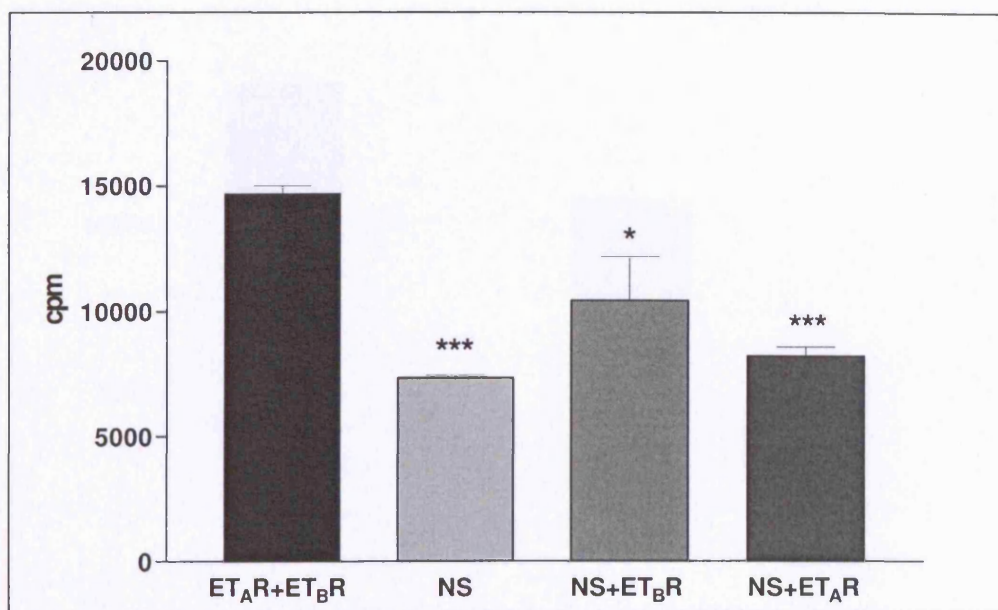


Figure 4-2 *Binding of $^{125}\text{I-ET-1}$ to NRK-49F cells.* Membranes from NRK-49F cells were incubated with $^{125}\text{I-ET-1}$ (200pM). Bound radioactivity was measured in the presence of: 1 μM unlabelled ET-1 (NS), 100nM BQ485 (NS+ET_BR), 100nM BQ788 (NS+ET_AR) or with no competitor (ET_AR+ET_BR). The difference between ET_AR+ET_BR and NS represents the total specific binding to both receptors. Total radioactivity added gave a count of 16,900 cpm. The data shown are the mean values of $n=3\pm\text{SEM}$ except for ET_AR+ET_BR and NS groups where $n=4$. *, $p<0.05$; ***, $p<0.0001$.

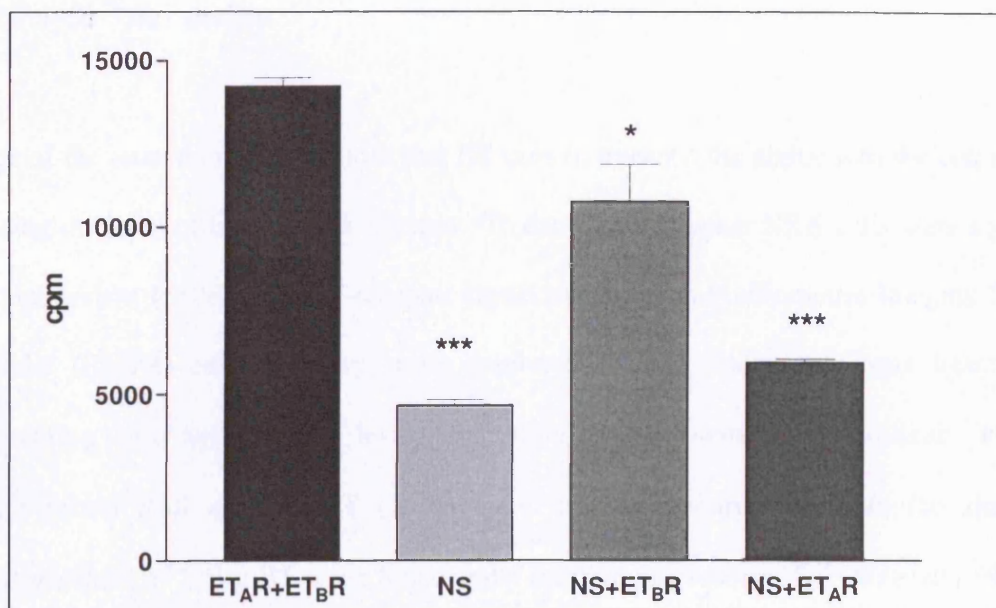


Figure 4-3 *Binding of ^{125}I -ET-1 to HeLa cells.* Membranes from HeLa cells were incubated with ^{125}I -ET-1 (200pM). Bound radioactivity was measured in the presence of: 1 μM unlabelled ET-1 (NS), 100nM BQ485 (NS+ET_BR), 100nM BQ788 (NS+ET_AR) or with no competitor (ET_AR+ET_BR). The difference between ET_AR+ET_BR and NS represents the total specific binding to both receptors. The data shown are the mean values of $n=3\pm\text{SEM}$. *, $p<0.05$; ***, $p<0.0001$.

4.2.2 Characterization of functional activity of ET receptors – signal transduction

4.2.2.1 Ca^{2+} flux studies

One of the most common methods that ET uses to transmit the signal into the cell is by raising the level of intracellular calcium. To determine whether NRK cells were a good model system for studying ET-receptor signal transduction Fluorometric Imaging Plate Reader (FLIPR) calcium assays were conducted. The experiments were based on measuring the changes in Ca^{2+} levels after stimulating the cells with endothelin. In the experiments PBS and Big-ET (1 μM) were used as negative controls (no rise in intracellular Ca^{2+}). Big-ET is the functionally inactive precursor of ET (Xu *et al.*, 1994). A positive control, ATP was also used.

Confluent quiescent cells were loaded with Fluo-4, a cell permeable calcium indicator dye ($\lambda_{\text{excitation}} = 488 \text{ nm}$, $\lambda_{\text{emission}} = 520 \text{ nm}$) before being stimulated. The cells were stimulated at a time point of 30 seconds, and fluorescence was measured every 2 seconds for the first 5 mins and then every 5 seconds for the next 10 mins. All measurements were repeated six times. The results of this study revealed increases in intracellular calcium in response to ET-1 (Fig. 4-4). In figure 4-4 panel A shows the effect of increasing concentrations of ET-1 on the intracellular Ca^{2+} signal. ET-1 was administered to the cells in three different concentrations: 10nM, 100nM and 1 μM . A 10nM concentration of ET-1 was not enough to induce a strong Ca^{2+} signal, although there was a slight rise (panel A, Fig. 4-4,). 100nM and 1 μM ET-1 produced a Ca^{2+} response that saturated very quickly. The Ca^{2+} signal started 6 seconds after the cells were stimulated and peaked after 25 seconds. After this time the Ca^{2+} level gradually went back to normal and reached the baseline 4 mins after the stimulation started.

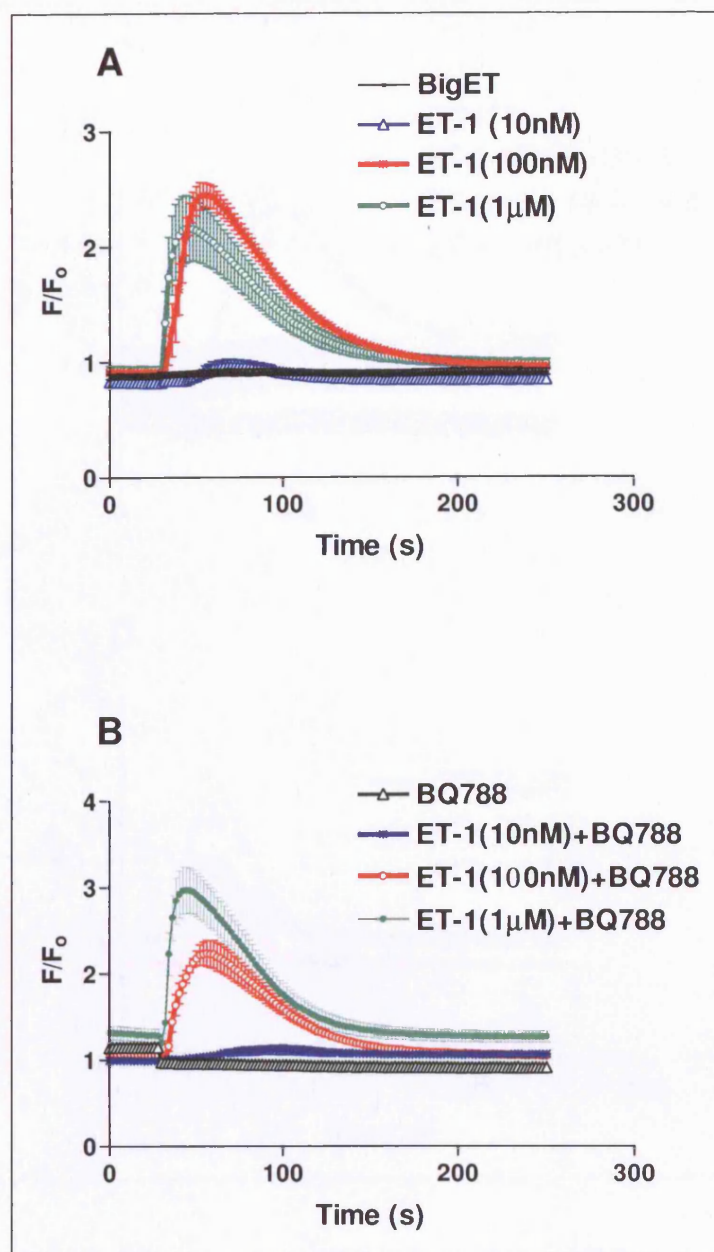


Figure 4-4 Ca^{2+} response in NRK cells to the application of ET-1. (Continued overleaf)

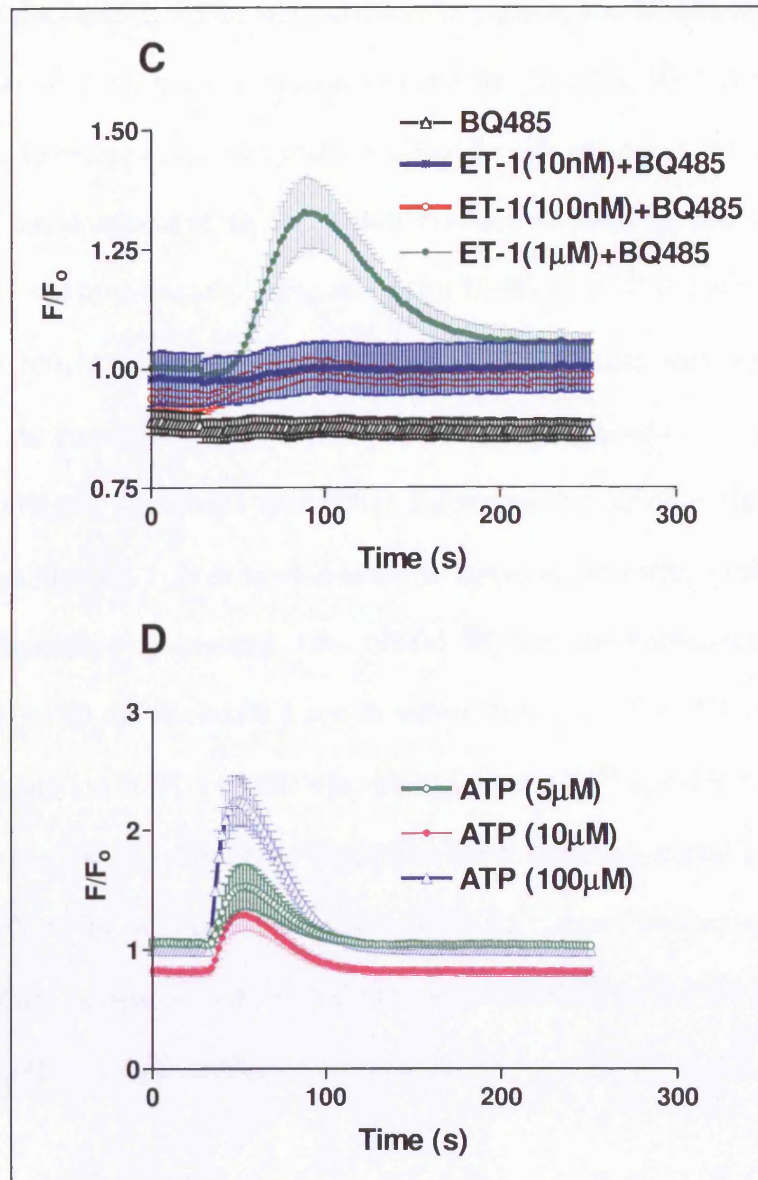


Figure 4-4 (cont'd) Ca^{2+} response in NRK cells to the application of ET-1. (A) A fluorescent imaging plate reader (FLIPR) assay measuring intracellular levels of Ca^{2+} was used to detect responsiveness of fluo-4 loaded NRK cells to ET-1. PBS and Big-ET were used as controls. (B) Ca^{2+} level was measured in the presence of ET_B receptor antagonist, BQ788 or, (C) in the presence of ET_A receptor antagonist, BQ485, after the induction of cells with increasing concentrations of ET-1. (D) ATP was used as a positive control for the assay. Data are the average of $n=6 \pm$ SEM. Measurements were carried out with the help of Mr S. Ranasinghe (Dept. of Physiology, UCL).

In panel B of the same figure the same effect as in panel A was monitored but this time in the presence of a constant concentration of BQ788 (100nM). As BQ788 selectively binds to the ET_B receptor and chemically modifies it with no agonist activity (Ishikawa *et al.*, 1994), the remainder of the Ca²⁺ signal would result from the activity of the ET_A receptor. As seen from the graph (Fig. 4-4 panel B) 10nM ET-1 did not induce a Ca²⁺ response, but 100nM and 1μM did induce a response. Data from this experiment were compared to the corresponding data from panel A using a paired t-test. A statistically significant difference was found (p<0.0001) suggesting that although the signal exists when ET_BR is blocked, it is somewhat lower in intensity than without the antagonist. When ET_A receptor was blocked with 100nM BQ485, the administration of ET-1 (10nM and 100nM) did not cause a rise in intracellular Ca²⁺ (Fig. 4-4 panel C). The highest concentration of ET-1 (1μM) was able to induce a Ca²⁺ signal in the presence of ET_AR antagonist, but the start of the signal was much slower: it started 1 min after the addition of ET-1. In addition, the intensity of the Ca²⁺ signal was significantly lower (p<0.0001) when compared to the signal intensity induced by 1μM ET-1 in the absence of antagonists (Fig. 4-4 panel A).

4.2.2.2 Activation of MAP kinase cascades in NRK-49F cells induced by ET-1

It has been suggested that the MAPK pathway is involved in the mitogenic activity of endothelins (Cazaubon *et al.*, 1993; Simonson *et al.*, 1992)). To assess whether MAPK signalling pathways are activated by ET in NRK-49F cells, antibodies which recognise the phosphorylated form of several members of the MAPK signalling pathway family were used. Changes in phosphorylation over time for p42/44 MAPK, ERK1/ERK2 (p38MAPK) and c-Raf 1 were followed not only in the whole cell extracts but also in the Immobilized Metal Ion Affinity Chromatography (IMAC) - enriched

phosphoprotein fractions (Figs. 4-5, 4-6 and 4-7). ET-1 (100nM) stimulated extensive phosphorylation of both ERK1/ERK2 (Fig. 4-5) and p38MAPK (Fig. 4-6). The activation of these kinases was rapid and peaked within 2 mins for ERK1/ERK2 and within 5 mins for p38MAPK. Phosphorylation of both MAP kinases remained above basal levels at 20 mins.

ET-1 stimulated phosphorylation of JNK was also analyzed with the phospho-SAPK (stress activated protein kinase)/JNK antibody. This antibody detects endogenous levels of p46 and p54 SAPK/JNK dually phosphorylated at threonine 183 and tyrosine 185. No changes in phosphorylation were observed for SAPK/JNK in either total cell extract or phosphoprotein enriched extract.

The effect of ET-induced phosphorylation on Raf was also observed (Fig. 4-7). Again the activation of this protein happened within 5 mins of inducing the activation of the receptors. Phosphorylation of Raf was observed only in the enriched phosphoprotein fraction. In the total cell extract the phosphorylation of this protein could not be detected.

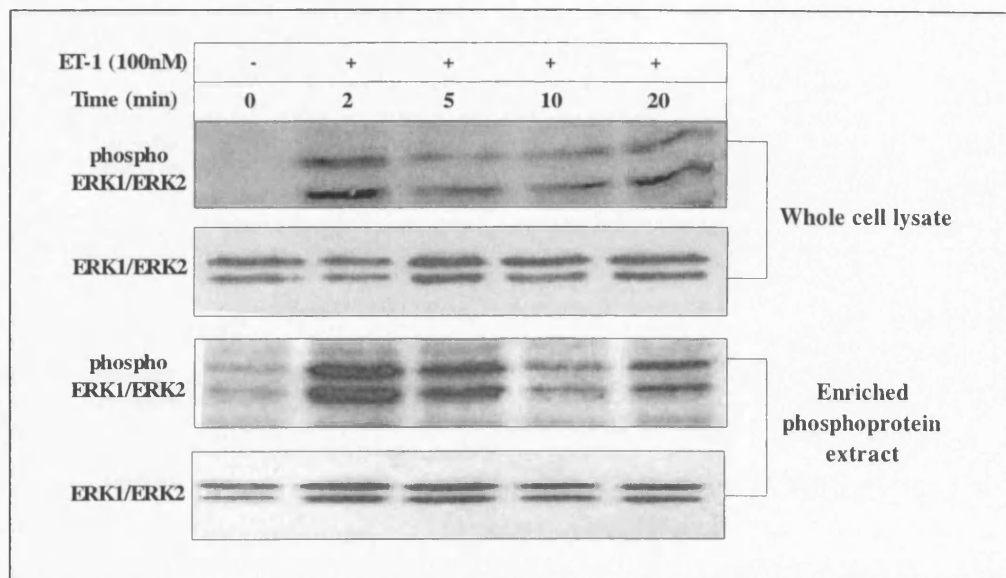


Figure 4-5 *Effect of ET-1 on the phosphorylation of ERK1/ERK2.*

Quiescent NRK cells were treated with or without ET-1 (100nM) for the indicated period of time. Proteins were separated on SDS-PAGE either immediately after cell disruption, or after the purification of phosphoproteins using IMAC. Gel separated proteins were transferred onto a nitrocellulose membrane and incubated with either anti-ERK1/ERK2 or antiphospho-ERK1/ERK2 antibodies. After the incubation with the primary antibody membranes were incubated with secondary anti rabbit antibody and protein bands were detected with a chemiluminescence detection kit. The images are the representative images of three individual experiments.

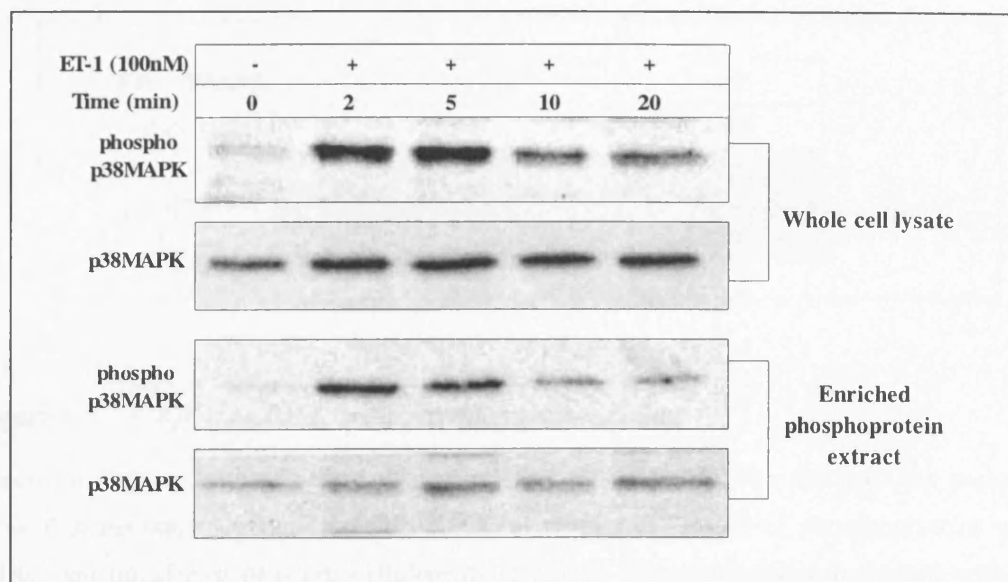


Figure 4-6 *Effect of ET-1 on the phosphorylation of p38MAPK.*

Quiescent NRK cells were treated with or without ET-1 (100nM) for the indicated period of time. Proteins were separated on SDS-PAGE either immediately after cell disruption, or after the purification of phosphoproteins using IMAC. Gel separated proteins were transferred onto a nitrocellulose membrane and incubated with either anti-p38 MAP Kinase or anti-phospho-p38 MAP Kinase antibodies. After the incubation with the primary antibody the membranes were incubated with secondary anti-rabbit antibody and protein bands were detected with a chemiluminescence detection kit. The images are the representative images of three individual experiments.

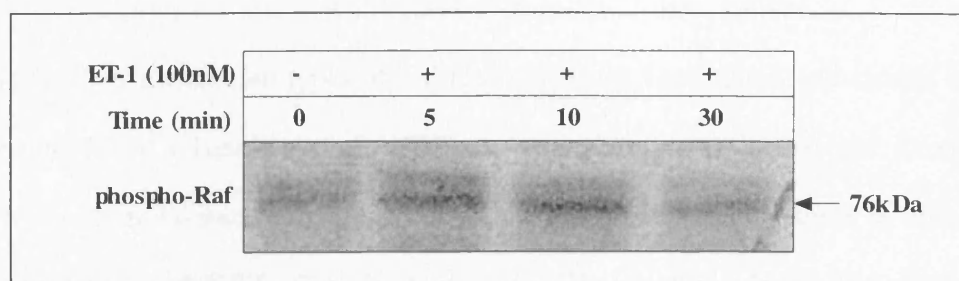


Figure 4-7 *Effect of ET-1 on the phosphorylation of Raf.*

Quiescent NRK cells were treated with or without ET-1 (100nM) for the indicated period of time. Proteins were separated on SDS-PAGE after the purification of phosphoproteins using IMAC and transferred onto nitrocellulose membranes. Membranes were incubated with anti phospho-Raf antibody and subsequently with secondary anti-rabbit antibody. Signals were detected using alkaline phosphatase detection.

4.3 Discussion

4.3.1 Binding studies for testing the expression of ET receptors

In mammalian tissues two types of ET receptors have been found and cloned so far: ET_AR and ET_BR (Haendler *et al.*, 1992) and these are widely distributed throughout different cells and tissues. There has been speculation about the existence of other subtypes of ET_AR and ET_BR (Endoh *et al.*, 1998; Gellai *et al.*, 1996) but to date the experimental data supporting these findings have only been of a pharmacological nature.

ET_A and ET_B receptors can be present in any given tissue together or alone. Initial binding experiments were carried out in order to find a model cell system for the study of the ET receptor signalling pathways. RMVEC, NRK-49F and HeLa cell lines were tested for the presence of the receptors for ET using radioactively labelled ¹²⁵I-ET-1. Total radioligand binding was measured first. After this, the radiolabelled ligand was displaced by the cold ligand and whatever radioactivity was left over after the displacement was attributed to non-specific binding. The difference between total and non-specific binding provides the specific binding to ET receptors. RMVEC cells did not show a significant difference between the total bound radioactivity and the non-specific binding (Fig. 4-1). These results suggest that these cells do not express ET receptors. In both NRK-49F and HeLa cells the difference between the total and the non-specific binding was significant (***, $p < 0.0001$) indicating the presence of ET receptors (Figs. 4-2 and 4-3). To test whether both receptor types were present in NRK-49F and HeLa cells, antagonists that specifically block the receptor subtypes were used. The results from these experiments showed that both ET_AR and ET_BR are present in

both cell lines (Figs. 4-2 and 4-3). A statistical evaluation of these results indicated that the ET_BR is the predominant receptor type in both NRK-49F cells and HeLa cells ($p < 0.0001$). Earlier binding studies of different endothelins indicated the majority of ET receptors in NRK-49F to be of the ET_AR type (Yumet *et al.*, 1995). However, binding studies made by others showed that ¹²⁵I-ET-1 binding was displaced by unlabelled ET-2 in a similar dose response indicating the presence of both ET_AR and ET_BR in NRK-49F cells (Kusuhara *et al.*, 1992). In addition, results from our laboratory confirmed the presence of both ET_AR and ET_BR in NRK-49F using specific ET receptor antibodies (see Posters and Publications). The identity of the proteins recognised as ET_AR and ET_BR, and the specificity of the corresponding antibodies were further proved by MALDI-TOF MS.

Reports regarding the expression of ET receptors on HeLa cells are different. Karne *et al.*, (1993) reported that binding studies on HeLa cells showed no presence of ET receptors. Binding experiments by another group showed that both ET receptors are present in HeLa cells with ET_AR being the predominant type (Drimal *et al.*, 2000). Takahashi *et al.*, (2002) demonstrated weak mRNA expression of both ET receptors in HeLa cells.

The recent production of several specific ET_AR and ET_BR antibodies has facilitated the study of ET receptors by allowing the use of Western Blot and immunoprecipitation techniques. These techniques will allow a more direct quantification of receptors in different cell types and in cells exposed to different stimuli than techniques based solely on binding assays.

4.3.2 Testing the response of NRK cells to ET-1 stimulation

4.3.2.1 Ca^{2+} flux studies

It is known that ET-1 starts its signal in the cell after binding to the G protein-coupled receptor, which in turn activates G proteins of the $\text{G}_{q/11}$, $\text{G}_{12/13}$ and G_i families (Aramori *et al.*, 1992; Takigawa *et al.*, 1995). ET receptors have been associated with different G proteins depending on the cell type where they are expressed. The most common feature of the ET signalling cascade is its ability to increase the levels of intracellular calcium. ET-1 does so through the activation of PLC (see Chapter 1), which hydrolyses PIP_2 to IP_3 and DAG (Pollock *et al.*, 1995). IP_3 binds to specific receptor ion channels on the membrane of the endoplasmic reticulum and initiates the release of the Ca^{2+} stored in this organelle. Both types of ET receptors were shown to be able to elevate Ca^{2+} levels in the cell upon binding of the ligand (Touyz *et al.*, 1995; Hyvelin *et al.*, 1998).

In order to test the responsiveness of NRK-49F cells to ET-1, measurements of the level of intracellular calcium were carried out after stimulating the cells with ET. These experiments were performed using FLIPR. The cells were first pre-loaded with a Fluo-4 calcium indicator dye and were then treated with different agonists or antagonists. The dye indicates the change in Ca^{2+} concentration via its fluorescent spectral change upon binding to Ca^{2+} (Minta *et al.*, 1989). PBS and Big-ET were used as controls.

The Ca^{2+} signal was monitored after treating the cells with three different concentrations of ET-1: 10nM, 100nM and 1 μM . The lowest concentration of ET-1, 10nM was not able to give a strong Ca^{2+} signal, but higher concentrations succeeded in elevating the level of intracellular calcium to a maximum after 6 seconds (Fig. 4-4, panel A). The

intensity of the signal peaked 25 seconds after stimulation and then gradually reached basal level after approximately 5 mins. These results agree with previous data reporting that ET-1 induces PLC and therefore the increase in Ca^{2+} in NRK cells (Lahaye *et al.*, 1999; Kusuvara *et al.*, 1992).

ET_A and ET_B receptor antagonists were then used to see which of these two receptor subtypes generates the Ca^{2+} signal in NRK-49F cells. As seen in panel B of Fig. 4-4, the ET_A receptor was able to stimulate the calcium response in a similar fashion to that displayed when no receptor antagonists were present. However, the signal induced by ET_AR alone (panel B) is lower in intensity ($p < 0.0001$) than the full Ca^{2+} signal (panel A).

ET_B receptor alone was not able to induce the Ca^{2+} signal (Fig. 4-4, panel C). There are several possible explanations for these results. One explanation could be that the downstream signalling of the ET_B receptor is not coupled to the signalling of Ca^{2+} in this type of cell. 1 μM concentration of ET-1 was able to induce a small and slow calcium response when the ET_A receptor was blocked. However, this could result from the fact that in such large quantities ET is able to push the antagonist out of the receptor and thereby induce a signal through the ET_A receptor. A second explanation is that the ET_A and ET_B receptors function together to give a full rise in intracellular calcium. Recently demonstrated constitutive or ligand induced oligomerization of several GPCRs (Pagano *et al.*, 2001; Terillon *et al.*, 2004; Balasubramanian *et al.*, 2004; Guo *et al.*, 2003) has also led to the discovery that ET receptors can form both hetero- (ET_AR-ET_BR) and homodimers (ET_AR-ET_AR and ET_BR-ET_BR) that are constitutively expressed (Gegan *et al.*, 2004 a, b). Perhaps the heterodimerization of ET receptors is necessary for enhanced signalling in certain cases like the one described in this chapter.

In summary the results of the calcium measurement experiments showed that both receptors together activate a strong signal that mobilizes the intracellular pools of calcium in NRK-49F cells and that the ET_A receptor alone is capable of inducing a calcium response in these cells.

4.3.2.2 Effects of ET-1 on MAP kinase cascades in NRK-49F cells

The role of ET in activating the three MAPK cascades has been studied extensively in a variety of cells (Simonson *et al.*, 1992; Cazaubon *et al.*, 1993; Clerk *et al.*, 1998). It has also been shown that ET-1 has cell proliferative effects in NRK-49F cells (Yeh *et al.*, 1991). The results presented in this chapter showed that ET-1 activates two MAP kinases: ERK1/ERK2 and p38MAPK (Figs. 4-5 and 4-6) in NRK-49F cells. ET-1 induced phosphorylation of ERK1/ERK2 happened within the first 2 mins of cell stimulation (Fig. 4-5). This effect decreased later (15 mins) but was still present even after 30 mins. Phosphorylation of Raf was also observed (Fig. 4-7) after 5 mins of cell stimulation with ET-1. This result is expected, as Raf is upstream of ERK1/ERK2 in this signalling cascade. ET-1 could activate the c-Raf/ERK cascade in NRK-49F cells via several distinct mechanisms:

- by Gq activation of PKC (via PLC) (Kolch *et al.*, 1993);
- by activation of Ras (via Gβγ subunit) (Herman and Simonson 1995); or
- by arrestin induced mobilization of Src to the GPCR, which in turn could trigger the signal leading to ERK (Luttrell *et al.*, 1999; Hall *et al.*, 1999).

It is also possible that the activation of the ERK1/ERK2 pathway occurs as a result of the transactivation of the EGF receptor as has been demonstrated in previous reports (Daub *et al.*, 1996; Hua *et al.*, 2003). In this way ET receptors borrow the signalling

molecules from the tyrosine kinase receptor cascade to fulfil their mitogenic function. This kind of connection seems very probable when considering results from other groups showing that ET-1 stimulation increased the number of EGF receptors in the membranes of NRK cells (Kusuhara *et al.*, 1992; Yeh *et al.*, 1991). In addition, ET-1 induced anchorage-independent growth of NRK cells which was regulated by EGF (Yumet *et al.*, 1995; Lahaye *et al.*, 1999).

Similarly to ERK1/ERK2, ET-1 influenced the phosphorylation states of p38MAPK (Fig. 4-6). p38MAPK was activated within the first 2 mins of cell stimulation. A second wave of phosphorylation was not observed for this enzyme over the studied period of time. Although it has been shown that ET-1 can induce the p38MAPK pathway (Clerk *et al.*, 1998), the exact mechanism of this activation is still not fully understood.

The phosphorylation of JNK was also analyzed within the same time period of ET-1 stimulation in both whole cell extracts and enriched phosphoproteins. No changes were detected with the antibody used (phospho SAPK/JNK antibody from Cell Signaling Technology).

4.4 Conclusions

- NRK-49F cells express both ET_A and ET_B receptors (ET_B>ET_A);
- ET-1 induced the rise of intracellular calcium predominantly through the activation of the ET_A receptor in NRK-49F cells;
- ET-1 stimulated the activation of the Raf/ERK cascade; and
- ET-1 stimulated the activation of p38MAPK.

CHAPTER 5. ET-1 INDUCED PHOSPHORYLATION CHANGES IN THE PROTEOME OF NRK-49F CELLS

5.1 Introduction

The binding of ET to ET_AR and ET_BR leads to the activation of G proteins (of the G_{q/11} and G_{12/13} families) which is followed by the activation of a variety of downstream effectors (Clerk *et al.*, 1998). Amongst the induced kinase cascades are members of the MAPKs, PKC and Tyr kinase signalling pathways (Cazaubon *et al.*, 1993; Clerk *et al.*, 1998; Yamauchi *et al.*, 2002). The activation of these pathways transmits ET-1's signal to the nucleus where the transcription of protooncogenes is initiated. This results in cell growth and mitogenesis (Nelson *et al.*, 2003).

In NRK cells ET-1 has transforming and proliferative activity (Kusuhara *et al.*, 1992; Yeh *et al.*, 1991). In these cells the binding of ET-1 to ET_AR results in the mobilization of intracellular calcium and induces a rapid onset of second messenger production (Yeh *et al.*, 1991; Lahaye *et al.*, 1999). In addition to this, ET_AR activation was followed by the transcription of early response genes c-jun, jun B, and c-fos (Yumet *et al.*, 1995). ET-1 stimulated anchorage-independent cell growth and complemented the growth factor induced DNA-replication and G1/S-phase transition in NRK cells (Kusuhara *et al.*, 1992; Yeh *et al.*, 1991; Yumet *et al.*, 1995). In addition to this, ET-1 induced the up-regulation of EGF receptors in these cells (Yeh *et al.*, 1991; Yumet *et al.*, 1995).

With growing recognition of the importance of the role of ET-1 in tumour progression, a detailed understanding of the signal transduction pathways which influence the

malignant transformation of cells is of considerable interest and NRK cells provide an excellent study-model.

Protein phosphorylation is important for the regulation of the activity of proteins in a wide variety of cellular functions including signal transduction, metabolism and apoptosis (Pawson and Nash, 2003; Hunter, 1995). One third of the proteins expressed in mammalian cells are phosphorylated at serine, threonine and, less commonly, tyrosine residues (Krebs, 1994). Phosphoproteins separated on gels can be followed by:

- Radiography after ^{32}P radiolabelling (Kang *et al.*, 1997; Immler *et al.*, 1998);
- Immunostaining with phospho-specific antibodies (Stannard *et al.*, 2003 b; Steen *et al.*, 2002; Soskic *et al.*, 1999 a); and
- Fluorescent phosphoprotein dyes (Ge *et al.*, 2004; Schulenberg *et al.*, 2004; Patton, 2002).

In addition to the above methods, immobilised metal-ion affinity chromatography (IMAC) is one of the most efficient approaches for the extraction and enrichment of phosphopeptides. The oxygen atoms in the phosphorylated side chains of serine, threonine and tyrosine, as well as carboxylic acids, show a high affinity for hard metal ions such as Fe(III) and this has been exploited to isolate phosphopeptides under acidic conditions (Chaga, 2001). Substantial progress has been made with this approach and its application as a reliable method for the analysis of phosphorylated peptides by mass spectrometry (Neville *et al.*, 1997; Jin *et al.*, 2004; Liu *et al.*, 2004; Nuhse *et al.*, 2003).

In an attempt to gain an insight into the regulatory mechanisms of ET signal transduction pathways the global effect of ET-1 on the phosphoproteome of NRK-49F cells was analysed. The qualitative, quantitative, and kinetic changes in the

phosphorylation states of the proteins were assessed in the enriched phosphoproteome of rat kidney fibroblasts following brief stimulation with ET-1. Enrichment was achieved by a Fe^{3+} coupled IMAC methodology developed in our laboratory. The results presented in this chapter show that ET-1 modifies the phosphorylation status of different protein groups. This indicates that the mechanisms ET-1 uses to propagate its signal in NRK cells involve regulatory proteins and enzymes and that the mechanisms include gene expression, the modulation of the cytoskeleton and molecular chaperones and energy metabolism.

5.2 Results

5.2.1 *Two-dimensional phosphoproteome maps of NRK-49F cells*

To detect rapid changes in phosphorylation states of proteins, synchronized NRK-49F cells were stimulated for 2 and 4 mins with ET-1. The phosphoprotein fraction was selectively purified from the whole cell lysate by IMAC complexed with Fe^{3+} . The obtained phosphoproteins were then separated according to their differential pI values on a 13 cm IPG strip (150 μg /strip, pH range 4-7). After the separation in the first dimension, the strips containing proteins were loaded onto the second dimension, SDS-PAGE, which allowed additional separation according to molecular weight. The phosphoprotein 2D maps of NRK-49F cells were detected by staining the gels with silver (Fig. 5-1). Approximately 600 protein spots on a single gel were reproducibly detected after silver staining.

The phosphoproteome maps showed reduced complexity of pattern compared to the protein pattern observed on 2D maps of total cell-proteomes. The map is also unique in

the sense that it contains only the phosphoproteins, making their detection much simpler. To assess the changes in differential phosphorylation states of proteins between the unstimulated and stimulated cells, gel images were analysed with Melanie III software. As the experiment was performed in triplicate, the software created a so called “synthetic gel” that represented the average gel out of triplicate sets of gels for each experimental condition (unstimulated and stimulated cells). A comparison of intensities was then performed between these average “synthetic” gels obtained from unstimulated and ET-1-stimulated NRK cells. For each spot, normalized volumes (the volume of the spot divided by the total volume over the whole gel, where the volume of the spot represents the integration of the optical density of the spot over the spot’s area) were calculated for each of the experimental conditions. From the results of the quantification analysis of the spots in the gels in Fig. 5-1, a number of quantitative changes in the intensity of the phosphoproteins were observed (see Fig. 5-2 and Fig. 5-3 for examples of enlarged gel-sections and Fig. 5-4 for kinetic diagrams of phosphorylation changes).

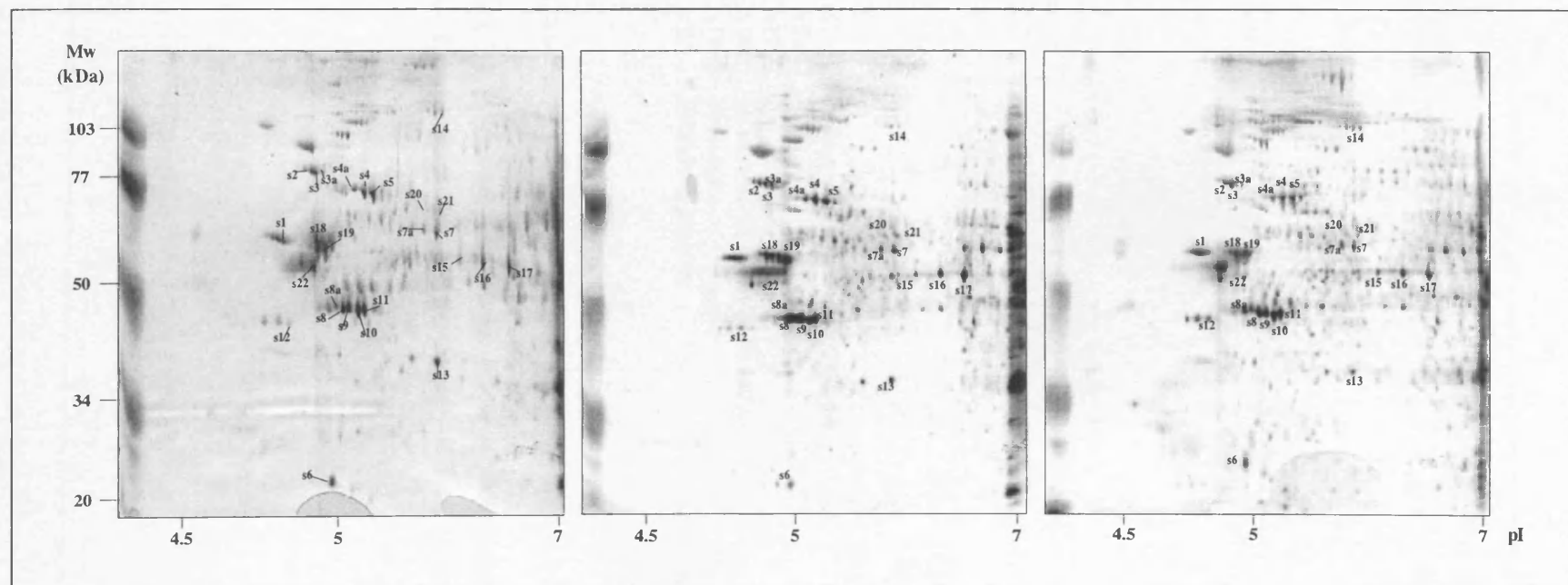


Figure 5-1 *Quantitative changes in the total phosphoproteome of NRK-49F cells after stimulation with ET-1.*

Quiescent NRK-49F cells were treated with ET-1 (100nM) for 0, 2 and 4 mins. After cell lysis the phosphoprotein fraction was enriched using an IMAC column and was separated by 2D PAGE. Protein spots were detected with silver staining. Spot intensities were analysed in Melanie software for quantitative changes. The proteins that were identified with MALDI-TOF peptide fingerprinting are labelled on the gels. Spot identification numbers correspond to Table 5-1. Enlarged parts of the gels are illustrated in Fig. 5-2 and Fig. 5-3. The gels shown are representative of a single experiment repeated three times.

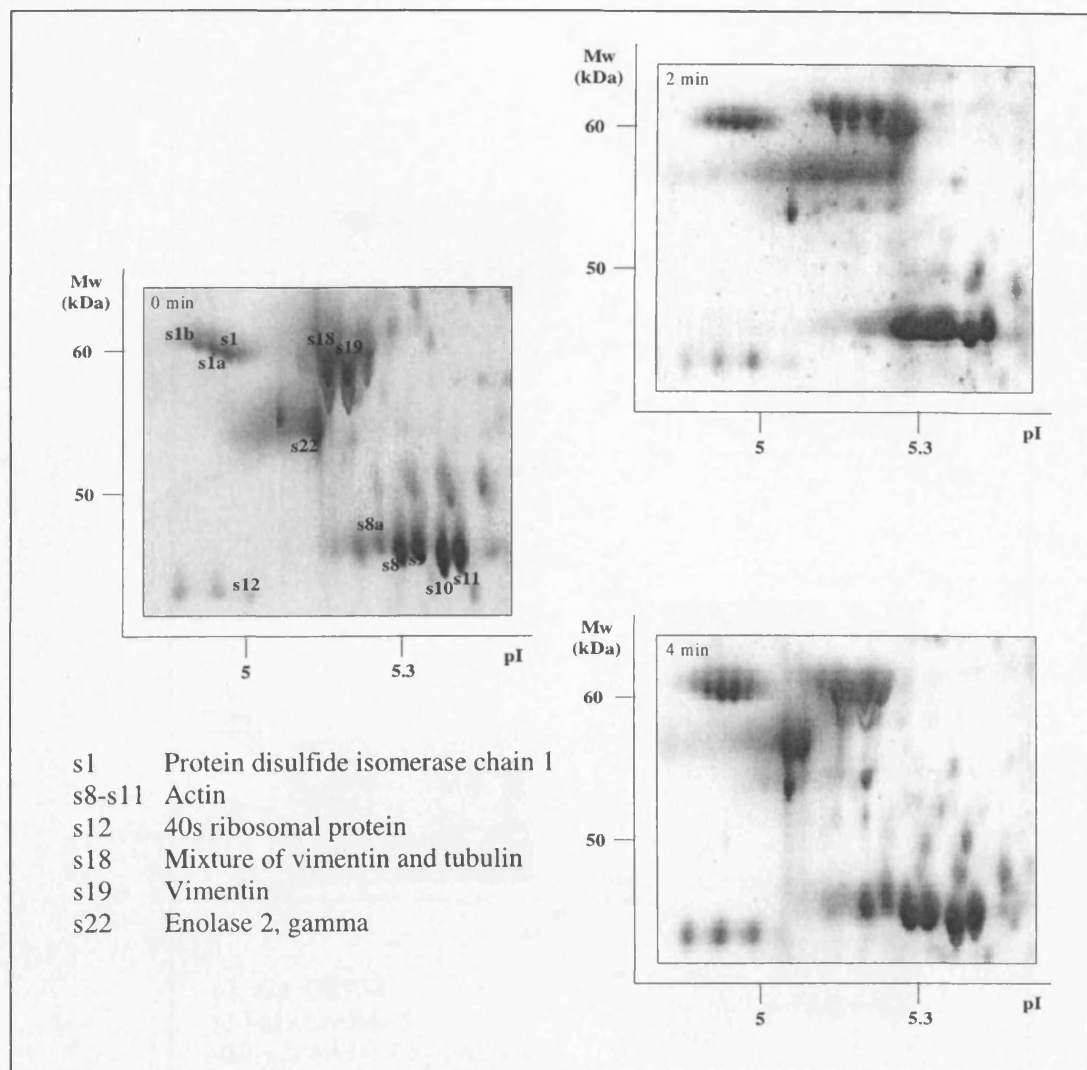


Figure 5-2 *Quantitative changes of protein phosphorylation in NRK-49F stimulated with ET-1.* Enlarged parts of the gels shown in Fig. 5-1 are illustrated.

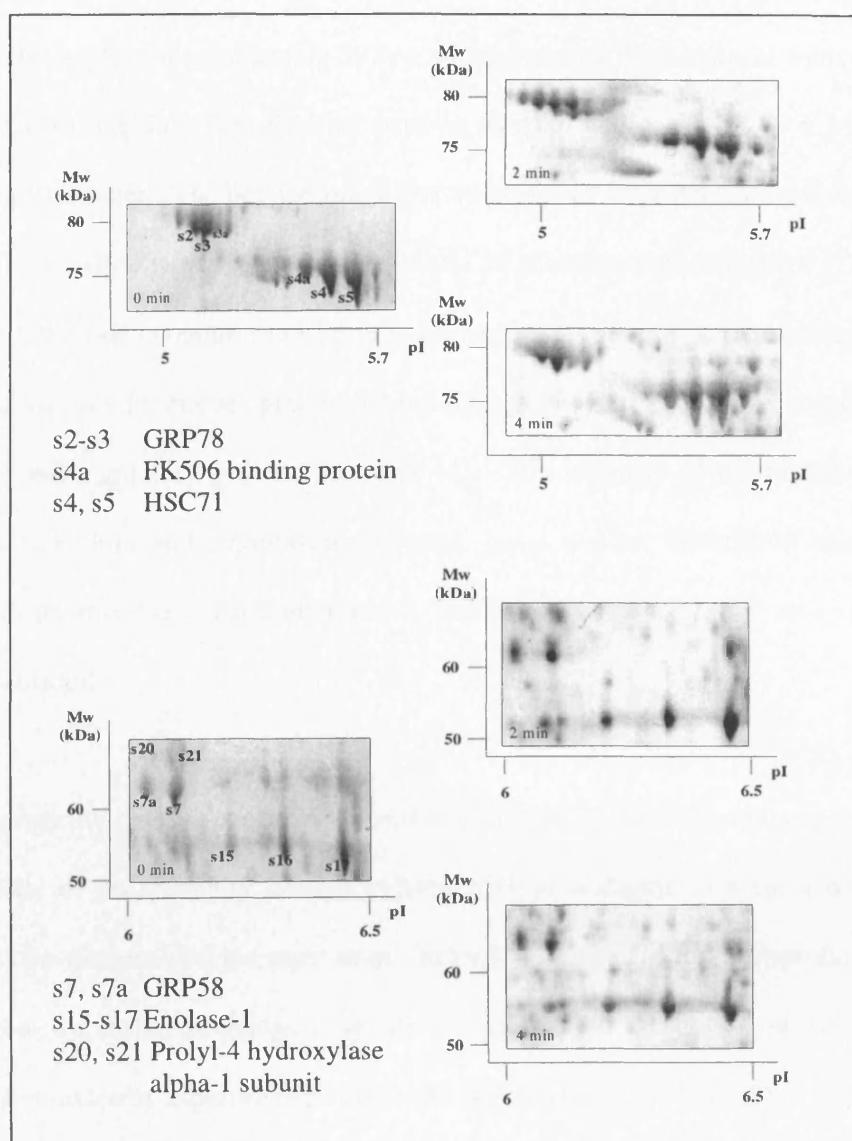


Figure 5-3 *Quantitative changes of protein phosphorylation in NRK-49F stimulated with ET-1.* Enlarged parts of the gels shown in Fig. 5-1 are illustrated.

Spots that showed >20% change between at least two of three experimental conditions (untreated cells and cells treated for 2 and 4 mins with ET-1) were selected for analysis by mass spectrometry. Approximately 30 spots that satisfied these criteria were cut out and digested with trypsin. The resulting peptide mixture was analysed by a MALDI-TOF mass spectrometer. The peptide mass spectra analysed with Bruker Data Analysis software were searched in sequence databases and 25 proteins were identified (Table 5-1). 8 of the identified proteins were folding accessory proteins, with the remaining 17 belonging to various functional groups, including cytoskeletal proteins (7), metabolic enzymes (4) and regulatory proteins/enzymes (4). The majority of the proteins were endoplasmic reticulum and cytoplasmic proteins, but a nuclear (interferon regulatory factor 1) and an integral plasma membrane (calcium-transporting ATP-ase) protein were also identified.

It is also noteworthy that some of the proteins were found in several protein spots. It is a characteristic of differentially phosphorylated protein isoforms to form a train-like pattern on a two-dimensional gel separation. Indeed, a number of these phosphoprotein isoforms showed a different change in intensity compared to the other isoforms of the same protein at different experimental conditions (Fig. 5-1 spots s1 and s8).

Spot	Gene ¹	Protein	GI ²	Accession ³	Closest ref. ⁴	Mw theo.	Mw ⁵	pI theo.	pI ⁶	No. of pept. ⁷	% coverage ⁸	Source ⁹	Comments ¹⁰	Score ¹¹
1. Cytoskeletal proteins														
s8	Actb	beta-actin	13592133	NP_112406.1		42	48	5.3	5.3	7	20	Rat		76
s8a	Actb	beta-actin	13592133	NP_112406.1		42	50	5.3	5.2	10	31	Rat		138
s9	Actb	beta-actin	13592133	NP_112406.1		42	50	5.2	5.3	13	42	Rat		105
s10	Actb	put. beta-actin (aa 27-375)	49868	CAA27396.1	34856512; 13592133	39	47	5.8	5.5	14	40	Mouse	Rat; cytoplasmic β-actin	86
s11	Actg	gamma-actin	809561	CAA31455.1	34856512; 13592133	52	48	5.6	5.5	13	45	Mouse	Rat; cytoplasmic β-actin	103
s18	Vim	vimentin	14389299	NP_112402.1		54	60	5.1	4.8	28	32	Rat	mixture	102
	Tubal	alpha-tubulin	11560133	NP_071634.1		51	60	4.9	4.8	15	46	Rat		71
s19	Vim	vimentin	14389299	NP_112402.1		54	57	5.1	5.0	27	30	Rat		282
2. Enzymes														
s15	Eno1	Enolase-1, alpha	6978809	NP_036686.1		47	52	5.8	6.2	9	26	Rat		70
s16	Eno1	Enolase-1, alpha	6978809	NP_036686.1		47	52	5.8	6.4	12	32	Rat		88
s17	Eno1	Enolase-1, alpha	6978809	NP_036686.1		47	52	5.8	6.5	14	38	Rat		113
s22	Eno2	enolase 2, gamma	26023949	NP_647541.1		47	50	5	5.2	14	39	Rat		135

Table 5-1 *List of phosphoproteins from NRK-49F cells modified by ET-1 and identified by mass spectrometry.* (Continued overleaf)

Spot	Gene ¹	Protein	GI ²	Accession ³	Closest ref. ⁴	Mw theo.	Mw ⁵	pI theo.	pI ⁶	No. of pept. ⁷	% coverage ⁸	Source ⁹	Comments ¹⁰	Score ¹¹
3. Folding accessory proteins														
s1	P4hb	Protein disulfide isomerase chain 1	202549	AAA40620.1		55	57	4.9	4.8	11	28	Rat		69
s2	HSPA5	78 kDa glucose-regulated protein precursor	25742763	NP_037215.1		72.5	70.5	5	4.9	19	33	Rat		174
s3	HSPA5	78 kDa glucose-regulated protein precursor	87528	A29821	25742763	72.2	70.5	5	5	23	39	Human	Rat (97%)	130
s4a	Fkbp10	Similar to 65kDa FK506 binding protein	34873708	XP_340902.1	6806907	65	46	5.4	5.2	12	29	Rat	Mouse; glycosylated	85
s4	HSPA8	Heat shock cognate 71kDa protein	123647	P19378	13242237	71	75	5.2	5.3	12	31	Hamsterr	Rat (99%)	157
s5	HSPA8	Heat shock cognate 71kDa protein	13242237	NP_077327.1		71	75	5.4	5.4	27	50	Rat		70
s7	GRP58	ER-60 protease	8393322	NP_059015.1		57	60	5.8	6	15	30	Rat		141
s7a	GRP58	ER-60 protease	8393322	NP_059015.1		57	60	5.8	5.9	20	42	Rat		145
s20	P4ha1	Prolyl 4-hydroxylase alpha-1 subunit	51036657	NP_742059.2		61	65	5.6	5.9	6	14	Rat		92
s21	P4ha1	Prolyl 4-hydroxylase alpha-1 subunit	33859596	NP_035160.1	25453386	61	65	5.6	6	16	33	Mouse	Rat (96%)	126

Table 5-1 (cont'd) *List of phosphoproteins from NRK-49F cells modified by ET-1 and identified by mass spectrometry.* (Continued overleaf)

Spot	Gene ¹	Protein	Gi ²	Accession ³	Closest ref. ⁴	Mw theo.	Mw ⁵	pI theo.	pI ⁶	No. of pept. ⁷	% coverage ⁸	Source ⁹	Comments ¹⁰	Score ¹¹
4. Regulatory proteins/enzymes														
s6	ARHGDI	RhoGDI-1	21759130	Q99PT1	34875656	24	21	5.1	5	9	50	Mouse	Rat	93
s12	LAMR1	40S Ribosomal protein SA (P40)	730681	P38983		33	40	4.8	4.8	8	36	Rat		67
s13	IRF1	Interferon regulatory factor 1 (IRF-1)	124902	P23570		37	38	5.1	6	10	23	Rat		78
s14	ATP2B2	Plasma membrane calcium transporting ATP-ase (PMCA2)	14286100	P11506		132.5	120	5.7	6	27	25	Rat		85

Table 5-1 (cont'd) *List of phosphoproteins from NRK-49F cells modified by ET-1 and identified by mass spectrometry.* 1. Name of the gene coding for the identified protein; 2. the NCBI sequence identifier; 3. accession number of the database record followed by a dot and a version number; 4. the Gi of the homologous rat/mouse sequence in cases where database search output retrieved sequences from organisms other than rat (see also 12), or in case where the difference in the observed and predicted Mw is too large; 5. Mw estimated from SDS-PAGE; 6. pI estimated from SDS-PAGE; 7. number of MS identified peptides in the database search; 8. percentage of protein sequence covered by the identified peptides; 9. organism species that the Gi sequence was retrieved from; 10. sequence homology to rat/mouse genome, percentage of homology is indicated where less than 99%; if homology is high, the explanation for the difference in observed mass is given where known; 11. the probability based Mowse score taken from Mascot; in Profound search all identified proteins had a P value 1.0e+00 and Est'd Z values were between 1.06 and 2.39.

5.2.2 *Phosphorylation kinetics*

Phosphorylation of 60% of the proteins identified (15 of 25) was increased after treatment with ET-1 (Fig. 5-4) for a brief period of time (0-4 mins). Phosphorylation levels of the proteins were examined at time points 2 mins and 4 mins. However, some of these increases were transient, with an increase observed after 2 mins of stimulation, followed by a return to the phosphorylation level in unstimulated cells at 4 mins (Fig. 5-4). 9 of the identified proteins showed an increase in phosphorylation after 4 mins of treatment with ET-1 (Fig. 5-4, panels A, B, C, F and H). The rest of the identified proteins underwent dephosphorylation after treatment with ET-1. Some of these phosphoproteins displayed a biphasic phosphorylation decrease, with an initial 2 mins decrease followed by either a return to the level of phosphorylation in the unstimulated cells by the 4th minute (Fig. 5-4, s17) or a further decrease after 4 mins (Fig 5-4, panels C, D, F, G and H).

Observation of the kinetic behaviour of cytoskeletal proteins (s8, s8a, s9, s10 and s11) revealed that all actin phosphoprotein isoforms are rapidly phosphorylated after 2 mins of ET-1 stimulation (Fig. 5-4 panel E). The other cytoskeletal protein, vimentin, showed different isoform behaviour. While one of the phospho-isoforms of vimentin (s18) underwent a brief dephosphorylation before returning to the control level after 4 mins of ET-1 treatment, the other phosphoisoform (s19) was further dephosphorylated after 4 mins (Fig. 5-4 panel G). This result is not however conclusive, as s19 was identified as a mixture of vimentin and tubulin, so the different phosphokinetic behaviour observed could result from either of the proteins.

Folding accessory proteins (78 kDa glucose regulated protein (GRP78), FK506 binding protein 10, heat shock cognate 71kDa protein (Hsc70), protein disulfide isomerase (PDI) and ER-60 protease) as a functional group, show variable changes in phosphokinetic behaviour, between themselves. In addition changes in the level of phosphorylation were observed between phosphoisoforms of a single folding accessory protein. The level of phosphorylation increases for all of them after ET-1 treatment, apart from FK506 binding protein 10, which is dephosphorylated (Fig. 5-4, panel H, s4a). GRP78 is present in the gel with five different phosphoisoforms (Fig 5-3) three of which were identified by MALDI-TOF MS (s2, s3 and s3a). s3 and s3a both undergo phosphorylation after ET-1 treatment, but the phosphorylation of s3a is transient returning to control-level after 4 mins (Fig. 5-4 panel B). The s2 phosphoisoform of GRP78 decreases in phosphorylation by approximately 1.4 times in relation to the control. ER-60 protease was identified in its two phosphoisoforms (s7 and s7a), with s7 transiently increasing in phosphorylation and s7a steadily decreasing (Fig. 5-4 panel D). Hsc70 phosphoisoforms (s4 and s5) also show opposite phosphokinetic behaviour (Fig. 5-4 panel C).

Two isoenzymes of enolase were also identified, enolase alpha and enolase gamma (Table 5-1). The presence of gamma enolase has been previously reported only in nervous tissues. Two phosphoisoforms of alpha isoenzyme (s16 and s17) decrease in phosphorylation after 2 mins, while s15 increases after 2 mins (Fig. 5-4 panel F).

Regulatory proteins, IRF-1 and RhoGDI, were also identified. The phosphorylation of RhoGDI is increased after 4 mins of ET-treatment, whereas IRF-1 undergoes dephosphorylation. The phosphorylation of plasma membrane-associated calcium-transporting ATPase was also found to increase after 2 mins of ET-1 treatment (Fig. 5-4

panel H, s14) as well as the 40s ribosomal protein (s12). This protein was present in the gel in several phosphoisoforms (Fig. 5-2) which were not identified in the experiments discussed in this thesis.

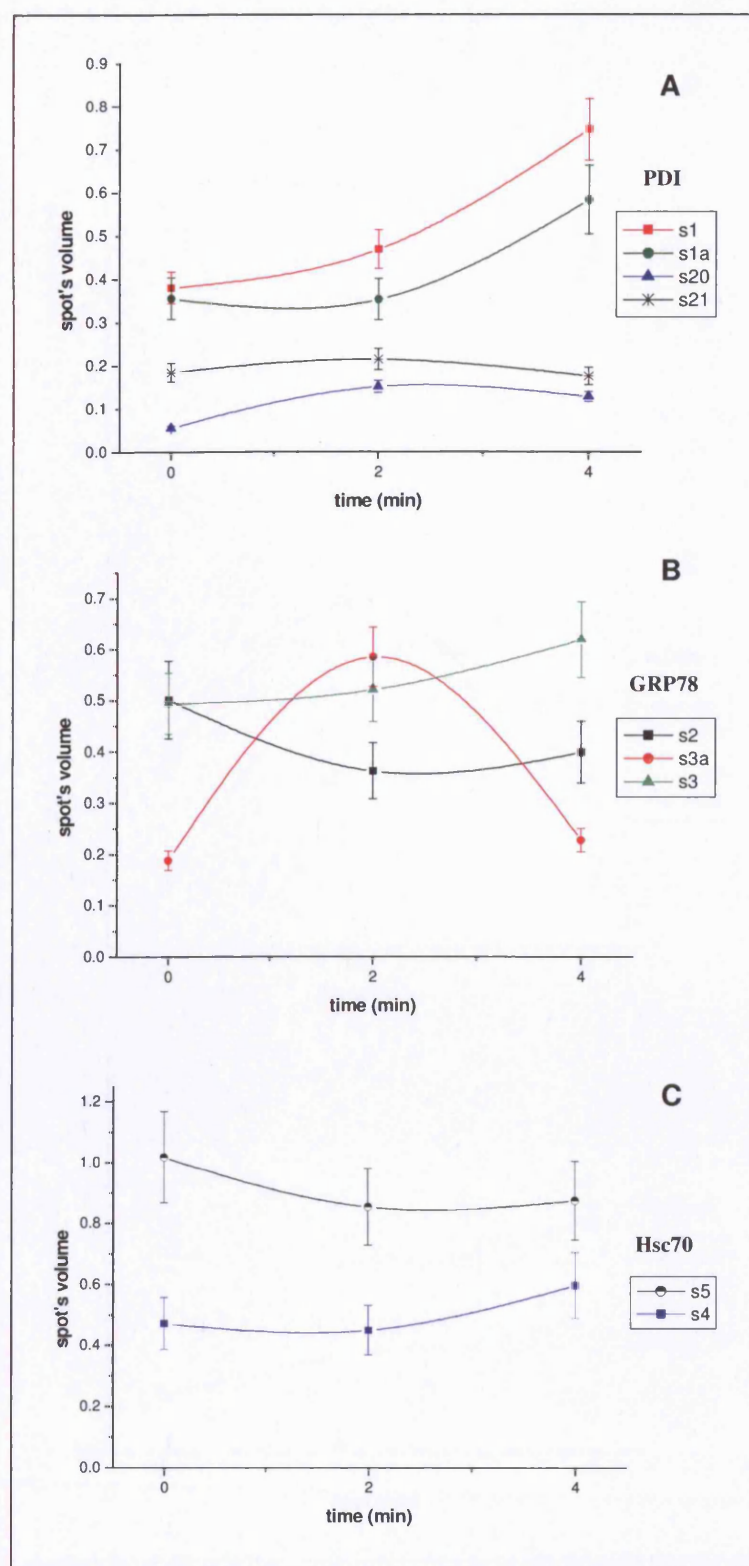


Figure 5-4 *ET-1* modifies protein phosphorylation in NRK-49F cells in a time dependent manner. (Continued overleaf)

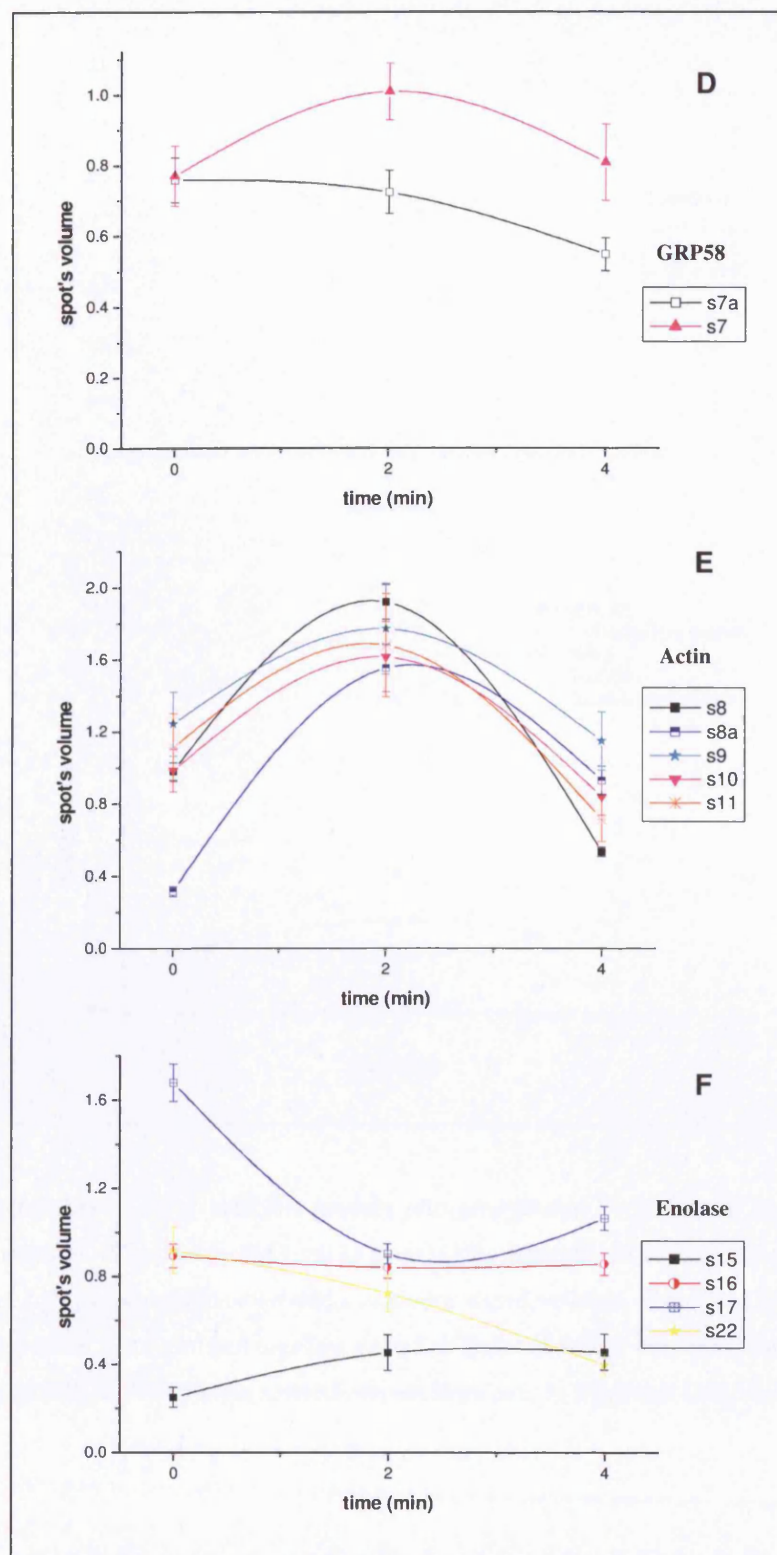


Figure 5-4 (cont'd) *ET-1* modifies protein phosphorylation in NRK-49F cells in a time dependent manner. (Continued overleaf)

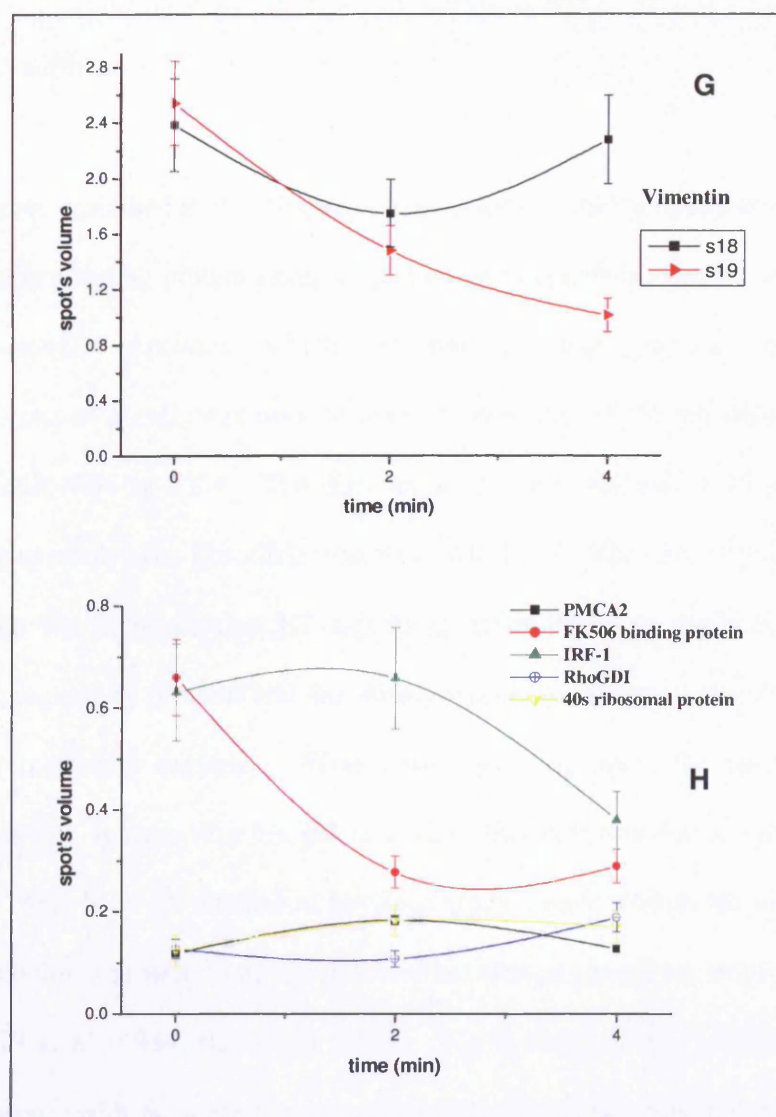


Figure 5-4 (cont'd) *ET-1* modifies protein phosphorylation in NRK-49F cells in a time dependent manner. Changes in the level of protein phosphorylation after treatment with ET-1 (100nM) for 2 and 4 mins were calculated using normalized volumes of spots. Charge isoforms of the same protein were grouped together except in panel H where only one isoform for each protein was identified. For protein annotations see Figs. 5-1, 5-2, 5-3 and Table 5-1.

5.3 Discussion

The experiments described in this chapter aimed to look at the rapid cellular response to ET-1 through monitoring protein phosphorylation and dephosphorylation changes. The global “top-down” approach, which followed a large portion of the total phosphoproteome of a cell, was used to give an overview of the signalling pathways induced in NRK-49F by ET-1. The differential phosphorylation of 25 proteins was demonstrated to result from fibroblast treatment with ET-1. The diversity of the protein groups in this list indicates that ET signalling takes place on multiple levels that together with regulatory proteins and enzymes, involve cytoskeletal proteins, chaperone proteins and metabolic enzymes. Within the protein groups, the majority of the individual proteins appeared on the gel in several isoelectric isoforms (Figs. 5-1, 5-2 and 5-3 and Table 5-1). A number of previous studies have interpreted the isoelectric shift of an individual protein to be the result of the altered phosphorylation state of that protein (Blenis *et al.*, 1984; Rabilloud, 2002). Whilst these studies looked at the level of total proteome (with no separation of unphosphorylated and phosphorylated proteins being performed), the research conducted in this chapter showed the changes in pre-enriched phosphorylated proteins (the unphosphorylated proteins were removed by pre-fractionation). This means that the proteins observed were already phosphorylated and that after stimulation with ET-1 the changes in spot intensity of each protein spot reflected the changes of a specific “phosphoisoform” of that particular protein. In brief, each of these isoforms showed specific changes in phosphorylation state, suggesting that each identified protein is composed of differently phosphorylated molecules.

The enrichment of the phosphoproteome of the cell was undertaken with a view to simplifying the pattern of spots analysed. In the control (unstimulated) gel there are

proteins that are phosphorylated already in the resting cell. Ideally a lot of newly phosphorylated proteins would be seen appearing in the gels representing stimulated phosphoproteome. Observing new spots proved to be difficult, and most of the observed proteins were still of low abundance (weakly detected on the gel with insufficient material to identify the spot). Pursuing the enrichment and identification of low abundance proteins is a very elaborate process but new and more sensitive methods that will aid this are on the horizon (Kleiner *et al.*, 2005, in press). In addition, not all multi-phosphorylated proteins will appear on the differential display. This will depend on the location of different phosphorylation sites in a protein i.e. if the phosphorylation is not on the surface of the protein, the probability of it binding to the IMAC column will decrease. Using Fe^{3+} IMAC methodology proteins containing pThr and pSer are enriched more than phosphotyrosinated proteins (Ficarro *et al.*, 2002; Ficarro *et al.*, 2003). As phosphotyrosine-proteome makes up 0.05% of the total phosphoproteome in vertebrate cells (Hunter, 1998), IMAC-enriched 2D maps of phosphoproteins will not cover this low abundant part of the phosphoproteome. Because of the facts stated above the experiments presented in this chapter focused on following the quantitative changes in phosphorylation of abundant phosphoproteins after treatment with ET-1.

5.3.1 *Cytoskeletal proteins*

ET stimulates cytoskeletal rearrangements in fibroblasts via the ET_A receptors (Gohla *et al.*, 1999). It has been shown that ET can induce the phosphorylation of several proteins involved in the formation of focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996; Salazar and Rozengurt, 1999; Leopoldt *et al.*, 2000). These results demonstrate how the link between ET and the cytoskeleton affects cell growth,

migration and contraction. In addition to this, actin organization has a role in occupancy (ET)-induced receptor internalization (Lunn *et al.*, 2000).

Three cytoskeletal components (actin, vimentin and tubulin) were identified the phosphorylation of which was altered in rat kidney fibroblasts after stimulation with ET-1. The initial increase in actin phosphorylation (Fig 5-4 panel E) may be the signal that leads to the reorganization of the actin-cytoskeleton. This is supported by studies from other groups where phosphorylation is implicated as a mechanism of regulation of the actin-cytoskeleton in different cells (Reiss *et al.*, 1996; Gu *et al.*, 2003). Another set of results (see chapter 6) implicates the reorganization/polymerization of actin in rat kidney fibroblasts after 5 mins of ET-1 treatment which is consistent with reports of ET-1 induced actin reorganization (Hirshman and Emala, 1999). Additionally, ET induced the expression of actin in VSMC (Hahn *et al.*, 1993) and phosphorylation/polymerization of actin was shown to be regulated by calcium (Constantin *et al.*, 1998). Previous findings demonstrated a Tyr-dephosphorylation of actin upon 2 mins ET-treatment of human lung fibroblasts (Stannard *et al.*, 2003 b). Experiments in this chapter followed the global phosphorylation state of actin, not focusing on specific phosphorylation sites. It may be that different phosphorylation sites behave differently after stimulation because actin can be phosphorylated on various residues: Tyr (Gauthier *et al.*, 1997), Thr (Furuhashi *et al.*, 2002) and Ser (van Delft *et al.*, 1995); or that the regulation of actin is cell type specific. A detailed mapping of actin phosphorylation sites would aid understanding of the complex effects of actin phosphorylation.

Vimentin is a major cytoskeletal phosphoprotein in fibroblasts that participates in building intermediary filaments (IF). Phosphorylation/dephosphorylation of vimentin

tightly regulates the balance between free vimentin and polymerized IF (Eriksson *et al.*, 2004). Vimentin was found to be a substrate for various kinases, amongst which are Rho-kinase (Inada *et al.*, 1999) and Aurora-B (Yasui *et al.*, 2004) both of which regulate the assembly/disassembly of vimentin filaments during cytokinesis. In interphase cells the activity of kinases on vimentin is counterbalanced by excessive phosphatase activity (Inada *et al.*, 1999; Turowski *et al.*, 1999). In this study, following the kinetics of vimentin phosphorylation after brief stimulation with ET-1, a decrease in phosphovimentin abundance was observed (Fig. 5-4 panel G) which is in accordance with earlier findings from our group (Stannard *et al.*, 2003 b). In addition to vimentin dephosphorylation, the report from Stannard *et al.*, suggested the activation of protein phosphatase 2A (PP2A) after ET-1 stimulation of a fibroblast cell line. The dephosphorylation of vimentin observed in these studies implies the presence of phosphatase activity and PP2A being a major interphase phosphatase of vimentin (Turowski *et al.*, 1999) could explain the results obtained. Vimentin was identified in two phosphoisoforms (s18 and s19, Figs. 5-1 and 5-3): s18 transiently dephosphorylated after 2 mins of ET-treatment and s19 steadily dephosphorylated throughout the analysed time-scale (Fig. 5-4 panel G).

The protein in spot s18 was identified as a mixture between vimentin and tubulin, preventing a clear distinction between the signals of the two individual proteins being made. Alternatively the signal might be the sum of the intensities of both proteins.

Tubulin is a major constituent of microtubules that regulate cell shape and polarity and are involved in cellular transport and mitogenesis. Tubulin isotypes undergo a variety of posttranslational modifications including phosphorylation. This process can be catalyzed by tyrosine kinases such as Syk in B lymphocytes (Faruki *et al.*, 2000),

pp60c-src in nerve growth membranes (Matten *et al.*, 1990) and Ser/Thr kinases like GRK2 (Pitcher *et al.*, 1998) and potentially Ca-CaM kinase (Goldenring *et al.*, 1984). The general thought is that the phosphorylation of tubulin negatively regulates its assembly into microtubules (Wandosell *et al.*, 1987). So far tubulin phosphorylation/dephosphorylation was not reported in ET induced cellular signalling. The results presented in this chapter showed tubulin dephosphorylation after ET treatment (Fig. 5-4 panel G). Based on the data from literature, this would mean that ET promotes tubulin incorporation into microtubules. The biological significance of this needs to be further analyzed as microtubules could have different roles in fibroblasts: cell motility (Magdalena *et al.*, 2003) mitogenesis (Ball *et al.*, 1992) and receptor mediated endocytosis (Kapeller *et al.*, 1993).

5.3.2 *Folding accessory proteins*

Proteins that have a chaperone-like activity and aid in the correct folding of proteins into their 2D/3D structure are located mostly in the endoplasmic reticulum. Members of this large group of proteins have been implicated in taking part in some cell signalling processes (Higuchi *et al.*, 2004; Guo *et al.*, 2002; Thulasiraman *et al.*, 1998), but knowledge of their role as potential signalling molecules is scarce. Many proteins from this group are phosphorylated. The purpose of such posttranslational modification definitely goes beyond serving to stabilize the complex formed between chaperones and their interacting proteins and remains to be discovered. There are no reports that show the involvement of folding accessory proteins in ET-signalling pathways. The findings from this chapter demonstrate for the first time the rapid phosphorylation changes of this protein group induced by ET-1. 5 types of proteins were identified that change their phosphorylation states after the ET-1 stimulation of NRK fibroblasts: protein

disulfide isomerase (PDI), GRP78, immunophilin FKBP65, ER-60 protease and HSC71 (Table 5-1). It is interesting to note that the prolyl 4-hydroxylase (P4H) α subunit was also identified, which together with PDI (the β subunit) forms a functional P4H enzyme. The phosphorylation of PDI, GRP78, HSC71 and FKBP65 was previously reported (Quemeneur *et al.*, 1994; Hendershot *et al.*, 1988; Coss *et al.*, 1995; Peake *et al.*, 1998). ER-60 protease can be speculated to be phosphorylated based on the sequence similarity it shares with its cognates (from the same protein family) which were observed to be posttranslationally modified by phosphorylation (Quemeneur *et al.*, 1994).

PDI was located in the 2D gels in three spots, two of which were identified with MALDI-TOF fingerprinting (Fig. 5-2; s1, 1a). s1 and s1a increase their phosphorylation after 2 mins and increase or remain at the same level of phosphorylation after 4 mins of ET-1 stimulation (Fig. 5-4, panel A). Isoelectric isoforms of PDI could be assigned to the phosphorylation of several different sites. PDI is phosphorylated by a Src-like tyrosine kinase Lyn on a few Tyr residues (Donella-Deana *et al.*, 1996) but was also found to be a substrate for sphingosine-dependent kinases (SDK), implying Ser/Thr phosphorylation (Megidish *et al.*, 1999). The role of PDI phosphorylation in ET signalling is unclear, but taking into account the fact that PDI is phosphorylated downstream of TNF (Yanagida *et al.*, 2000); that its phosphorylation is modulated in ischemic rats (Sakai *et al.*, 2003); that it regulates negatively NF-kappa B signal (Higuchi *et al.*, 2004); and is generally a multifunctional enzyme, forming complexes with different partners and being localized in different cell compartments (reviewed in Noiva, 1999); the participation of PDI in signalling processes becomes imminent.

ER-60 protease is another PDI, also known as GRP58. Spots s7 and s7a indicate ER-60 protease on the gel (Fig. 5-1). There are no reports on the phosphorylation of GRP58 or its involvement in ET signal transduction, but as it is a PDI, the likelihood of phosphorylation of ER-60 is increased. ER-60 is an important factor in cell signalling as demonstrated by: its association with interleukine activated Stat3 (Guo *et al.*, 2002); its ability to regulate sarco endoplasmic reticulum calcium ATPase (SERCA) (Li *et al.*, 2004); its role in the MHC class I assembly (Lindquist *et al.*, 2001); and the role it has in the degradation of proteins (Otsu *et al.*, 1995). In addition to these signalling processes, GRP58 could have a role in cancer (Celli *et al.*, 2003).

GRP78 is a member of the heat shock 70kDa protein family and is resolved on 2D gel in several charge isoforms (Fig. 5-1 s2, s3 and s3a) as demonstrated also by others (Leustek *et al.*, 1991; Witzman *et al.*, 1994). Spots s3 and s3a show dynamic kinetic behaviour, increasing in phosphorylation after ET-1 treatment (Fig. 5-4 panel B). GRP78 is phosphorylated on Ser and Thr (Hendershot *et al.*, 1988) and additionally is an *in vitro* substrate of Src kinase (Carlino *et al.*, 1994). It is also found to undergo Ca^{2+} -induced autophosphorylation (Leustek *et al.*, 1991). As demonstrated in chapter 4, ET-1 induces a strong calcium signal that could modify the phosphorylation of GRP78. GRP78 is usually phosphorylated when forming oligomers, and it is thought that monomeric protein is the unmodified and biologically active form of GRP (Freiden *et al.*, 1992). The results in this thesis are the first report of ET induced phenotypical change of GRP78. Which signalling role, if any, this change might have is yet to be elucidated.

FK506 binding protein 10 (FKBP65) (Fig 5-1, s4a; Fig. 5-4, panel H) is a peptidylprolyl isomerase, that accelerates the rate of the folding of proteins during protein synthesis.

This protein is a member of a multigene family of FK506 binding proteins, originally discovered because of their binding of the immunosuppressant drug FK506 (Harding *et al.*, 1989). FKBP65 is posttranslationally modified with glycosylation and phosphorylation (Coss *et al.*, 1995). This explains why this protein migrates on an SDS-PAGE gel at a higher Mw (75kDa) than expected (65kDa) (Fig. 5-1 and Table 5-1). ET-1 stimulation of NRK cells induced a 2-fold decrease in phosphorylation of FKBP65 (Fig. 5-4 panel H). This result implies an activity of a protein phosphatase. The biological role of the ET-induced dephosphorylation is not known. It may be that in this way the binding to different ligands/partners is regulated. FKBP65 binds to tropoelastin (Davis *et al.*, 1998) but is also found in tissues where this protein is not expressed (Patterson *et al.*, 2000) suggesting the presence of other binding partners.

Heat shock cognate 71kDa protein, a member of HSP70 proteins, was identified in spots s4 and s5 (Fig 5-1). After 4 mins of ET-1 stimulation, the more acidic s4 increased in phosphorylation by 1.3 times when compared to the control, while the intensity of s5 decreased by 1.3 times (Fig. 5-4 panel C). An assumption can be made that additional phosphorylation of the protein is shifting the abundance of molecules towards the left. Again the biological role of this event is unclear. HSP70 proteins were found to be phosphorylated on Tyr residues (Otto *et al.*, 2001), Thr (Peake *et al.*, 1998) and in modest amounts by casein kinase II (Shi *et al.*, 1994). Generally the processes of protein phosphorylation/dephosphorylation were frequently associated with studies involving HSP70 (Chu *et al.*, 2001; Polanowska-Grabowska *et al.*, 1997). In addition, HSP70 are often shown to regulate some signalling mechanisms (Carter *et al.*, 1997; Thulasiraman *et al.*, 1998) indicating that their role in cellular signalling is not insignificant.

5.3.3 Enzymes

ET-1 stimulation of rat kidney fibroblasts in these experiments resulted also in phosphorylation changes of two enzymes: prolyl 4-hydroxylase (P4H) and enolase. There are no previous reports of ET-induced phosphorylation of these enzymes. In addition, P4H and enolase-gamma were not reported to be phosphorylated by anything else. P4H is listed in the folding accessory protein group (Table 5-1) because it is a subunit of PDI.

Enolase was identified in these studies in 4 spots, three of which represent isoelectric (phospho) isoforms of alpha-enolase (s15, s16 and s17) and s22 which is gamma-enolase, also known as neural specific enolase (NSE). This glycolytic enzyme was found to be phosphorylated in cells transformed by the Rous sarcoma virus (Cooper *et al.*, 1983). Later reports showed that enolase is a substrate for the insulin receptor kinase (Sale *et al.*, 1987) and EGFR kinase (Reiss *et al.*, 1986) but mostly it is used as a marker of kinase activity for pp60c-src kinase (Cartwright *et al.*, 1993). The three isoelectric isoforms of alpha-enolase, show different phosphorylation kinetics (Fig. 5-4 panel F) with s15 increasing, s16 constant and s17 decreasing in phosphorylation states after ET-treatment. As these spots represent the differentially phosphorylated forms of the same protein, it is possible that the decrease observed for s17 accounts for the increase in phosphorylation of s15 (s16 could be an intermediate phosphoisoform). It can be speculated that the ET-1 induced change in phosphorylation of alpha enolase is a result of the activity of Src kinase or ET-induced EGFR transactivation (Bisotto *et al.*, 2001; Daub *et al.*, 1996) and that its potential biological significance is related to the increased rate of cell growth, proliferation and transformation (Simonson *et al.*, 1996; Lahaye *et al.*, 1999) of NRK cells.

The appearance of NSE in rat kidney fibroblasts is an interesting finding, because this enzyme is used as a neuronal tissue marker (Sakimura *et al.*, 1987). It is also found in platelets so the possibility of this enzyme isoform appearing in cells other than neuronal cells is not excluded (Kato *et al.*, 1983). Another explanation for this result could be that rat fibroblasts express a homologue of gamma-enolase that has not yet been described and could be generated by protein splicing (Shakib *et al.*, 2005).

5.3.4 *Regulatory enzymes/proteins*

Several regulatory proteins were identified in this study to modify their phosphorylation state after a signal trigger by ET-1. These proteins are: IRF-1, 40s ribosomal protein, PMCA2 and RhoGDI-1. All of the regulatory proteins were reported previously for phosphorylation with greater or lower confidence (RhoGDI-1). For most of them the role of phosphorylation was implied in the regulation of their protein function, but there are still no clear views on this aspect. 40s ribosomal protein, PMCA2 and RhoGDI-1 can be directly and indirectly linked to ET signalling although no reports on the participation of these particular proteins in the signalling cascade were published (apart from one in respect of 40s ribosomal protein (Wang and Proud, 2002)). IRF-1, on the other hand, is a completely new target of ET-1. Together the results obtained in this chapter could provide insights into the previously unknown effects of ET.

Phosphorylation of 40s ribosomal protein has been implied in the up-regulation of protein synthesis in interphase cells (Thomas, 2002), in tumour growth (Comolli and Albert, 1988) and cell growth (Volarevic and Thomas, 2001). 40s ribosomal protein is phosphorylated by a Ser/Thr kinase S6 kinase that is activated by various mitogens and growth factors. It was reported that ET-1 activates S6K and consequently the

phosphorylation of 40S ribosomal protein in a signal regulating ET-induced cell growth (Iwasaki *et al.*, 1999; Wang and Proud, 2002). The signal upstream of S6K could involve ET_AR and Ca²⁺ dependent EGFR transactivation (Iwasaki *et al.*, 1999); Ras and MEK-1 (Wang and Proud, 2002); and ET_BR, PKC, Raf-1 and MAP kinase (Terada *et al.*, 1995). In the experiments presented in this chapter ET-1 induced an increase in phosphorylation of 40s ribosomal protein immediately after 2 mins and this increase remained strong after 4 mins (Fig. 5-1 and Fig. 5-4 panel H) These findings agree with the previous reports and imply the stimulation of a pathway leading to S6K activation which would suggest that ET-1 triggers signals that regulate protein synthesis and cell growth in rat fibroblasts. The regulation of protein synthesis by ET-1 accords with the results from chapter 3.

Although only one phosphoisoform of 40s ribosomal protein was identified (s12, Fig. 5-1 and Fig. 5-4 panel H) there are two additional spots left (more acidic) of s12, which very probably correspond to the same protein. In support of this assumption is the observed differential phosphorylation of 40s ribosomal protein resolved on a 2D PAGE reported by others (Leader *et al.*, 1980; Blenis *et al.*, 1984) and the fact that this protein was demonstrated to have multiple phosphorylated sites (Katahira *et al.*, 1996).

Phosphorylation of IRF-1 was observed to decrease slightly after 4 mins of ET treatment (Fig. 5-1, s13; Fig. 5-4 panel H). IRF-1 is a transcription factor, the activation of which was implied in different cellular responses: antiviral/antibacterial; anti-proliferative; pro- and anti-apoptotic; inhibition of cell transformation; and immune responses (Kroger *et al.*, 2002). Both Ser and Tyr phosphorylation of IRF-1 were detected (Lin and Hiscott, 1999; Kautz *et al.*, 2001) but it is not clear in what respect this regulates IRF-1 activity (whether it affects the protein-protein interactions or the

binding of IRF-1 to DNA). In ET biology, the release of ET from vascular smooth muscle cells is regulated by cytokine induced IRF-1 activation (Woods *et al.*, 2003). Based on the current knowledge of ET-induced biological effects and the biological effects caused by IRF-1 activation, there are several possible signalling events that can link these two molecules:

- IRF-1 up-regulation of nitric oxide synthase (Kamijo *et al.*, 1994), which causes the production of NO, the partner of ET in the regulation of vascular homeostasis;
- Inhibition of apoptosis demonstrated individually for both molecules (Chapman *et al.*, 2000; Shichiri *et al.*, 1998). IRF-1 also has pro-apoptotic effects (Tamura *et al.*, 1995); and
- Regulation of cancer development; IRF-1 acts as a tumour suppressor (Tanaka *et al.*, 1994), whilst ET promotes tumour development (Nelson *et al.*, 2003).

The outcome of any of the possible connections between ET and IRF-1 highly depends on the way in which ET regulates the activity of IRF-1 (activation/inhibition). Unfortunately, no conclusions can be drawn as to what effect the ET-induced dephosphorylation of IRF-1 observed in these studies might have on the transcriptional activity of this transcription modulator.

Plasma membrane calcium transporting ATPase (PMCA) is a key enzyme that regulates intracellular calcium concentration. There are four different isoforms of this enzyme: PMCA1, PMCA2, PMCA3 and PMCA4. Additional diversity of the enzyme isoforms is achieved through alternative splicing (Strehler and Zacharias, 2001). PMCA is a phosphoprotein which could have Ser, Thr and Tyr phosphorylation (James *et al.*, 1989; Wang *et al.*, 1991; Dean *et al.*, 1997). In the experiments presented in this chapter the

PMCA2 isoform of the protein was identified. The phosphorylation of PMCA2 was up-regulated after 2 mins of ET-1 treatment (Fig. 5-4 panel H) and returned to slightly above the basal level of phosphorylation, after 4 mins of ET-1 treatment. As ET induces a strong calcium signal (chapter 4) it would be logical to assume that it negatively regulates the activity of PMCA at least in the first instance, in order to maintain a high intracellular calcium level to transduce the signal further. Many papers support this assumption (Jouneaux *et al.*, 1993; Jouneaux *et al.*, 1994; Yokomori *et al.*, 2003). In addition to this, a report on phosphorylation induced down-regulation of PMCA activity strengthens this hypothesis (Dean *et al.*, 1997).

What is interesting is that a specific isoform of the enzyme was identified to be regulated by ET-1 in NRK cells. PMCA2 is predominantly expressed in the brain, but certain splice variants are found in specific tissues (Strehler and Zacharias, 2001). These are for PMCA2 expressed in kidneys: 2w, 2x and 2b. It is likely that the alternative splice isoform of PMCA2 observed here is one of these three. During peptide mass fingerprinting, several different databases were searched and all of them identified the spot s14 as PMCA2. Only the SwissProt database specified alternative splice-variants, out of which the highest probability score was for the PMCA2 xb splice isoform (SwissProt accession number P11506-6) with 30% of sequence coverage. In addition, only the xb splice variant contains Arg¹¹⁴² and the peptide containing this Arg was identified with peptide mass fingerprinting (see Appendix 2). Most probably this is the variant observed in this chapter as it agrees with tissue specific distribution of PMCA2 splice variants, but these results need to be confirmed by sequencing. xb splice variant is a result of a combination of two alternatively spliced domains at N-terminal site A (x) and at C-terminal site C (b). So far the splice sites have only been studied independently (Adamo and Peniston, 1992; Keeton *et al.*, 1993). The existence of

different isoforms of PMCA suggests that their activity can be differentially regulated: they bind calmodulin with different affinities and they have distinctive kinase consensus sequences (Guerini, 1998). The results in this chapter emphasize that ET could regulate different isoforms of PMCA, which could involve specific signalling pathways and this creates an extremely interesting new field to investigate.

Another regulatory protein the phosphorylation of which was up-regulated upon ET stimulation, is RhoGDI-1 (s6, Fig. 5-1 and Fig. 5-4 panel H). This protein negatively regulates the activity of Rho/Rac small GTPases, by forming a complex with them and inhibiting the GDP/GTP exchange. The activity of Rho GTPases is regulated by the GTPase-GDI complex dissociation, which in turn is tightly controlled by an as yet unknown signal input. One of the proposed mechanisms of the regulation of complex dissociation is the phosphorylation of GDI proteins (Bourmeyster *et al.*, 1996; Gorvel *et al.*, 1998, Mehta *et al.*, 2001). In this way, potentially steric hinderance of residues important for complex formation would cause it to disassemble. The results obtained in this chapter, encourage the validity of this hypothesis. They also prompted further study of the phosphorylation of RhoGDI (see Chapter 6).

As RhoGDI-1 is a guanine dissociation inhibitor with a general binding specificity (it can bind RhoA, RhoB, Rac1, Rac2 and Cdc42) it is difficult to tell which of these molecules is activated by an ET generated stimulus. ET can stimulate the activation of RhoA, Rac1 and Cdc42 (Fleming *et al.*, 1996; Shi-Wen *et al.*, 2004; Miyamoto *et al.*, 2003) and signalling pathways engaging these GTPases may lead to cell growth, cell proliferation, cell migration and contractility (Cazaubon *et al.*, 1997; Kim *et al.*, 1997; Miyamoto *et al.*, 2003; Chrzanowska-Wodnicka and Burridge, 1996).

5.4 Conclusions

- ET-1 induced rapid quantitative phosphorylation changes in NRK-49F cells.
- 25 abundant proteins that showed >20% change after ET-1 stimulation were identified.
- Phosphoproteins influenced by ET-1 belong to distinct protein groups (regulatory enzymes/proteins, cytoskeleton, metabolic enzymes, folding accessory proteins) demonstrating that the ET-1 signal occurs on various levels, from the modulation of cytoskeleton activity to calcium signalling, protein synthesis and gene expression. Again, these results suggest that the activation of parallel signalling pathways occurs downstream of ET receptors.
- The majority of the identified proteins exist in the cell as multiply-phosphorylated forms and it is the phosphorylation state of these isoforms that ET-1 alters.
- Within a group of isoforms of one protein, phosphorylation changes were quite different indicating different regulation of the same group of molecules. This is in line with the idea that different protein isoforms may be sequestered within a cell and targeted for a different fate (Godovac-Zimmermann *et al.*, 2005).
- Amongst the phosphoproteins modified by ET-1, RhoGDI was selected to be analyzed further (Chapter 6) for the following reasons:
 1. it is a new phospho-target of ET-1;
 2. its phosphorylation was hinted at by other groups but phosphorylation sites are not known; and
 3. determining phosphorylation site(s) could provide clues to the regulation of the stability of Rho-RhoGDI complex, a key signalling point in many signalling pathways.

CHAPTER 6. ET-1 INDUCED CHANGES OF RhoGDI PHOSPHORYLATION STATES

6.1 Introduction

Several reports have demonstrated that ET induced cellular signalling involves the activation of Rho proteins in a range of different cells (Fujihara *et al.*, 1997; Sakurada *et al.*, 2001; Kim *et al.*, 1997; Teixeira *et al.*, 1999; Cazaubon *et al.*, 1997). These results suggest that Rho GTPases are important regulators in the biological effects of ET such as: cell contraction, cell migration and adhesion, and cell proliferation.

In fibroblasts ET-Rho connection enables the generation of contractile force (through myosin light chain (MLC) phosphorylation) (Yee *et al.*, 2001), nuclear signalling (Kim *et al.*, 1997), and stress fibre formation (through the FAK pathway) (Gohla *et al.*, 1999). Some data even propose that Rho may play a role in ET-mediated receptor endocytosis (Lunn *et al.*, 2000).

The Rho-GDP guanine nucleotide dissociation inhibitor (GDI) is a protein that controls the activity of Rho GTPases which are key signalling molecules in a multitude of biological processes (Bishop and Hall, 2000). Rho proteins have a lipid modification on their C-terminus and as such cannot remain soluble in the cytosol. They use RhoGDI as a mask that protects their vulnerable hydrophobic surface from the aqueous surroundings (see Fig. 6-1). In this GDP-bound form as a sort of a heterodimer (Rho-RhoGDI) they rest inactively in the cell. When Rho is activated it translocates from the cytosol to the plasma membrane where it no longer needs the protection of GDI. The heterodimer dissociates and the active GTP-bound form of Rho activates its specific

downstream effectors. The cycle of Rho is complete when it turns into its inactive GDP-bound state that associates with RhoGDI and returns to the cytosol.

The main regulatory features of GDIs would be:

- keeping the Rho GTPases soluble; and
- keeping the Rho GTPases inactive, by inhibiting their otherwise spontaneous exchange of GDP↔GTP.

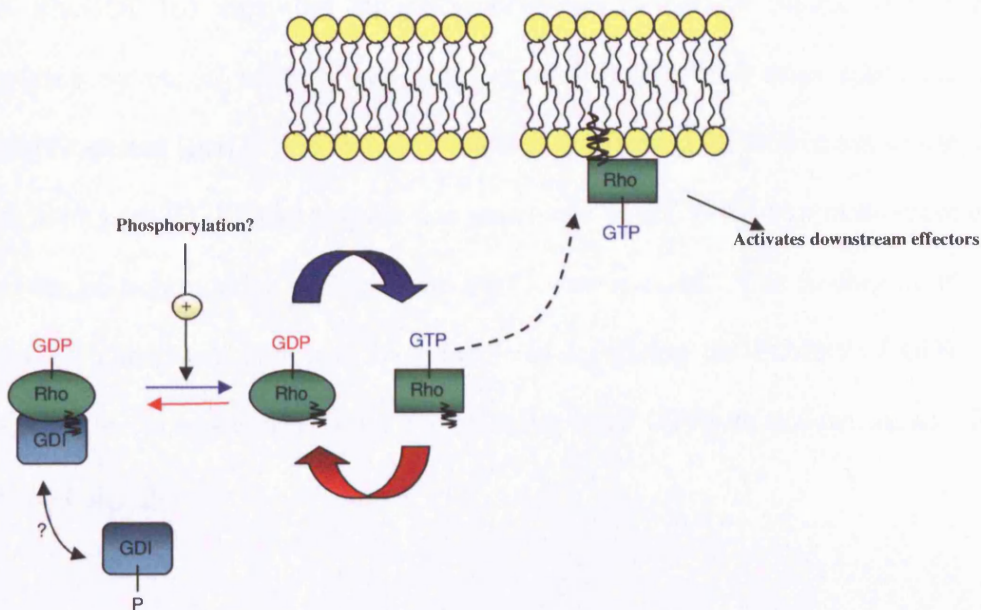


Figure 6-1 Cartoon showing the role of RhoGDI in the RhoGTPase activation cycle. See text for explanation of the processes illustrated in this figure.

The precise mechanism that causes the dissociation of the complex and therefore regulates the activation of Rho is still poorly understood. As RhoGDI has several motifs that are potential targets for different Ser/Thr kinases (see Fig. 6-11), it was proposed that phosphorylation may be one of the mechanisms that controls the stability of the Rho-RhoGDI complex. Previous reports gave indications of the possible phosphorylation of different RhoGDI isoforms (Bourmeyster *et al.*, 1996; Gorvel *et al.*, 1998; Mehta *et al.*, 2001) in different tissues.

Results from chapter 5 showed that the phosphorylation of RhoGDI increases after 4 mins of treatment with ET in rat kidney fibroblasts. To get a better understanding of the process of regulation of Rho GTPases, the phosphorylation of RhoGDI induced by ET-1 was analysed.

The studies of the phosphorylation of RhoGDI were extended by immunoprecipitating the RhoGDI and analysing the phosphorylation further by SILAC (stable isotope labelling by amino acids in cell culture) methodology and mass spectrometry. A phosphorylated RhoGDI peptide was detected in the MALDI-TOF mass spectrum after treatment with ET-1. This peptide was sequenced with ESI ion trap mass spectrometry, and the phosphorylation of Tyr¹⁴⁴ and Ser¹⁴⁸ was detected. This finding indicates that RhoGDI phosphorylation may have a role in regulating the stability of GDI-GTPase complex and consequently the activity of some small GTPases as downstream effectors of ET-1 signalling.

6.2 Results

6.2.1 Immunoprecipitation of RhoGDI

In the initial experiments NRK-49F cells were stimulated with ET-1 (100nM) for 2 and 5 mins and RhoGDI was immunoprecipitated from the cell lysate. The protein extract obtained after immunoprecipitation was resolved on SDS-PAGE and the RhoGDI was immunodetected with anti-RhoGDI antibody (Fig. 6-2 A). RhoGDI was resolved as a strong band at Mw~30kDa. The antibody detected another band (Mw~80kDa) that co-precipitated with RhoGDI after stimulation with ET-1.

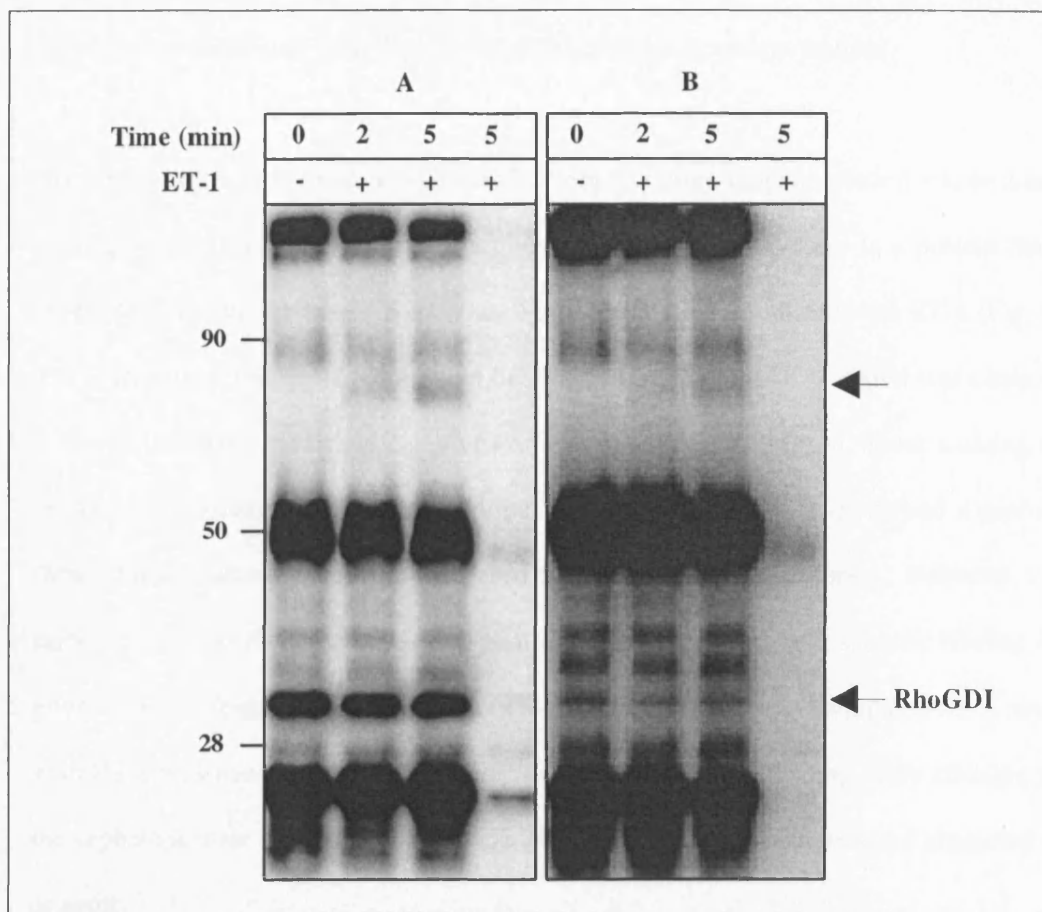


Figure 6-2 *Immunodetection of RhoGDI after ET-1 stimulation.* Quiescent NRK cells were treated with or without ET-1 (100nM) for the indicated period of time. RhoGDI was immunoprecipitated and the obtained protein extract was separated on SDS-PAGE. Gel separated proteins were transferred onto a nitrocellulose membrane and incubated with either anti-RhoGDI (A) or phospho(Ser)-PKC substrate (B) antibodies. After the incubation with the primary antibody membranes were incubated with secondary anti rabbit antibody and protein bands were detected with a chemiluminescence detection kit. The arrows indicate RhoGDI and the unknown protein (see text). The last column in both images represents the 'PA control' (the cell lysate proteins bound to protein A sepharose).

The same immunoblot was reprobed with phospho-(Ser) PKC substrate antibody (Fig. 6-2 B). Phospho-(Ser) PKC substrate antibody detected RhoGDI in both unstimulated and stimulated cell extract. Although it seems that there is dephosphorylation of RhoGDI after ET-1 stimulation, normalized values of the signal suggested that the

dephosphorylation was not significant. In addition the same antibody detected the protein co-precipitating with RhoGDI in a stimulation-dependent manner.

An interesting result came unexpectedly from the immunoprecipitation experiment: ponceau staining of the nitrocellulose membranes showed that there is a protein band (Mw~ 45kDa) that increased in abundance after cells were stimulated with ET-1 (Fig. 6-3 A). However, this protein could not be detected on the blot or its signal was covered by the signal from the heavy chain of immunoglobulin. In addition, silver staining of the gel with protein extract obtained after immunoprecipitation also showed a protein (Mw~ 45kDa) whose abundance increased 5 mins after ET-1 stimulation. However, the same protein was detected in the 'PA control' (used to monitor non-specific binding of proteins to the sepharose) (Fig. 6-3 B). Protein extract from cells stimulated for 5 mins with ET-1 was used for the 'PA control' and the protein was binding more strongly to the sepharose after stimulation. MALDI-TOF analysis of the protein band identified it as actin.

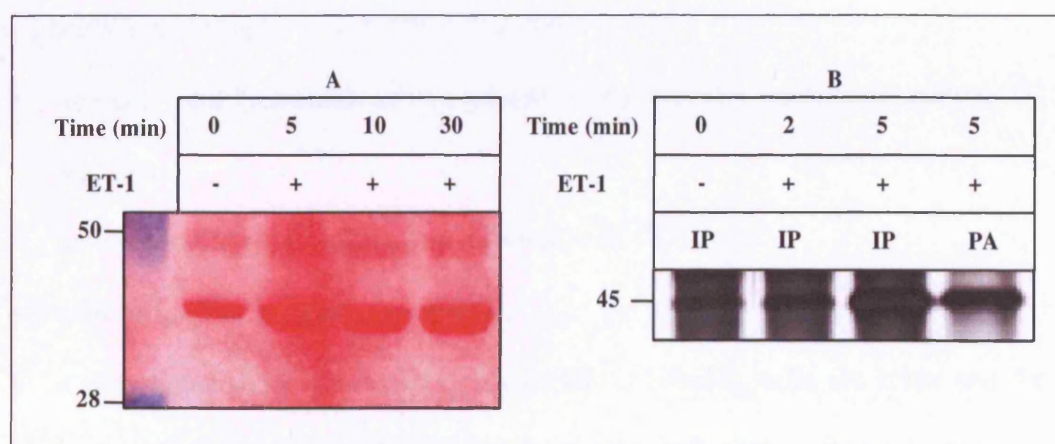


Figure 6-3 *ET-1 induced actin polymerization.* The experiment was performed as described in Fig. 6-1. (A) The nitrocellulose membrane stained with ponceau-S. (B) Gel separated proteins stained with silver. IP – immunoprecipitation; PA – control for the non-specific binding.

6.2.2 MALDI-TOF analysis of immunoprecipitated RhoGDI

To analyse the appearance of novel phosphorylated peptides in the RhoGDI molecule by mass spectrometry SILAC methodology was used. This novel method was described in studies of functional protein-protein interactions (Blagoev *et al.*, 2003) and quantitative estimation of protein abundance in cells (Ong *et al.*, 2002). The essence of the method lies in the application of two isotopes of the same element ^{12}C and ^{13}C (hydrogen and deuterium have also been used in SILAC experiments) (Ong *et al.*, 2002). ^{13}C is incorporated in arginine, an essential amino acid in cell culture (Scott *et al.*, 2000) which is then used to label proteins in the cell. The $^{13}\text{C}_6\text{-Arg}$ has a molecular weight 6 Da higher (because Arg has 6 carbon atoms in its structure) than the normal $^{12}\text{C}\text{-Arg}$, and this property (the mass difference) is later used in mass spectrometric analysis to distinguish peptides obtained from two differently treated cell pools (unstimulated and stimulated) (Fig. 6-6). The principle of the method used in the experiments in this chapter is as follows (Fig. 6-4):

- one cell pool (unstimulated) is grown on the medium containing normal ^{12}C arginine;
- the other cell pool (stimulated) is labelled with $^{13}\text{C}_6\text{-Arg}$;
- cells are grown in culture, as usual;
- a stimulation experiment is performed (ET-1, 100nM), cells are lysed and the protein concentration is determined from both cell pools. Equal amounts of proteins from unstimulated and stimulated cell lysates are mixed together (1:1);
- from the mixed lysates, RhoGDI is immunoprecipitated and separated on a 2D PAGE (Fig. 6-5); and

- RhoGDI spots are digested with trypsin and analysed with MALDI-TOF mass spectrometry to produce a characteristic peptide spectrum (Fig. 6-6); this spectrum contains doublet peak-pairs separated by 6 Da.

The aim of the experiments described in this chapter was to look for RhoGDI peptides phosphorylated after ET-1 stimulation. The SILAC methodology was applied with a view to detecting new peaks in the MS spectrum that represent phosphorylated RhoGDI peptides purified from the stimulated cell pool.

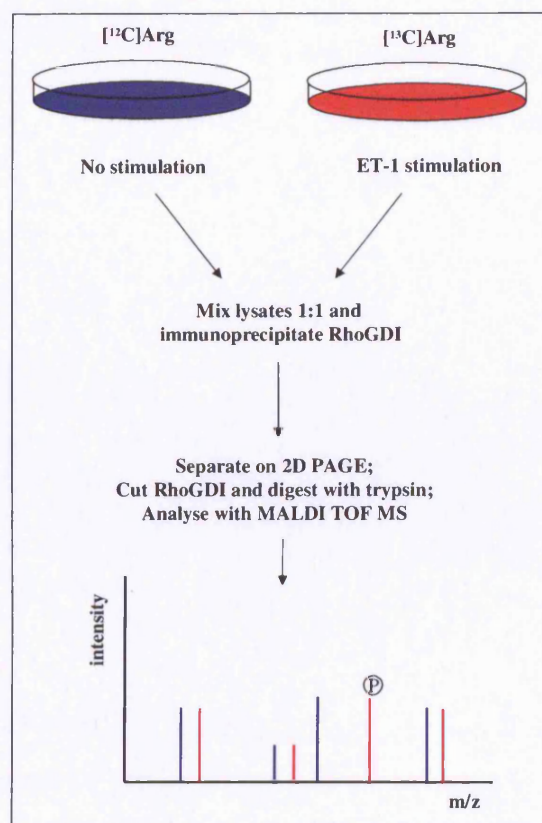


Figure 6-4 *A diagram of the SILAC method applied in this chapter.* In the mass spectrum peptide peaks from the control cell pool are blue; peptide peaks from the stimulated cell pool are red; the phosphorylated peptide peak is labelled with P.

2D gel separation of immunoprecipitated RhoGDI showed several spots in the Mw 25 kDa pI (4.9-5.3) coordinate range (Fig. 6-5). These spots were not present in either of the control gels. Three of them (s2, s3 and s4) were cut out, digested with trypsin and analysed with MS. Database searches of the obtained peptide masses identified all three spots as RhoGDI. The percentage of coverage of the RhoGDI sequence for spot s2 and s4 was 54% and for spot s3 it was 47%. Peptides that were identified for each of the spots are represented in Tables 6-1, 6-2 and 6-3.

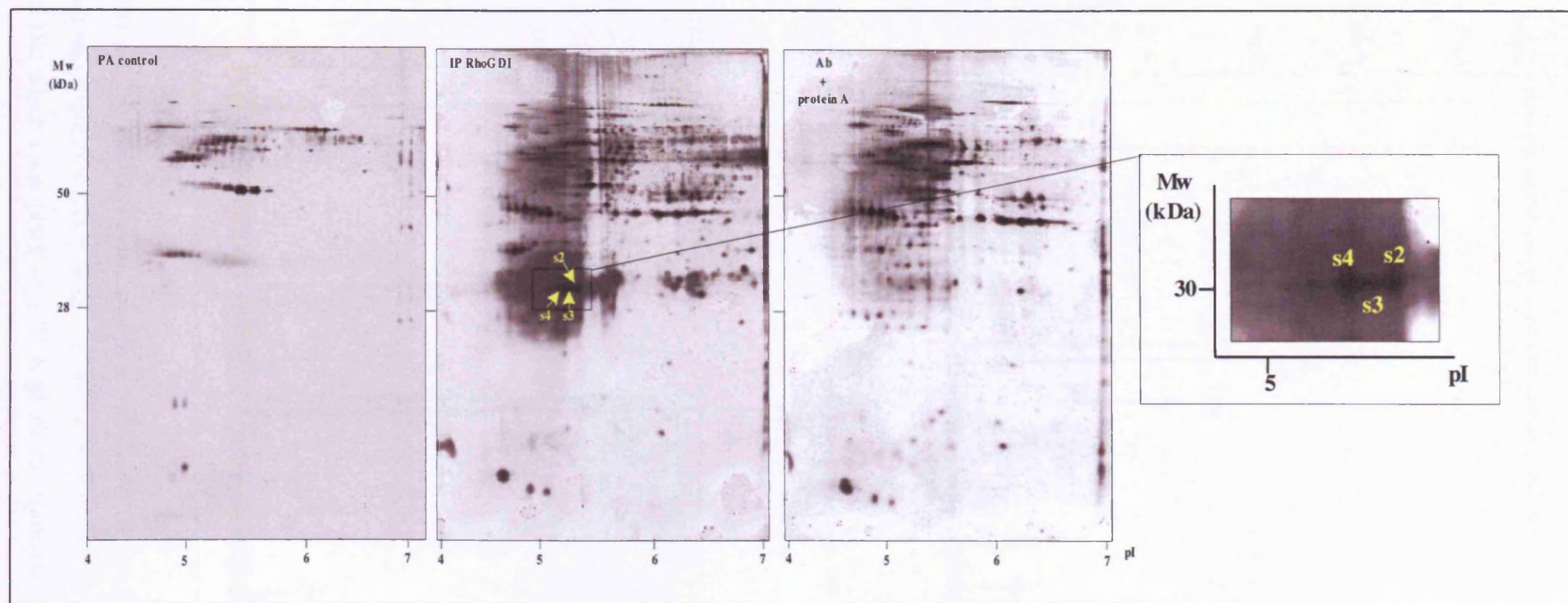


Figure 6-5 *2D PAGE separation of RhoGDI IP.* Cell lysates from unstimulated and [^{13}C] Arg labelled NRK-49F cells (labelled by Miss C. Guerrero), stimulated for 5 mins with ET-1 (100nM), were mixed 1:1 and incubated with anti-RhoGDI antibody. The yellow-labelled arrows show protein spots identified as RhoGDI with MALDI-TOF mass fingerprinting (Fig. 6-6). 'PA control' represents the cell lysate proteins bound to protein A sepharose. 'Ab+protein A control' illustrates the 2D PAGE separation of anti-RhoGDI antibody and protein A sepharose with no added cell lysate.

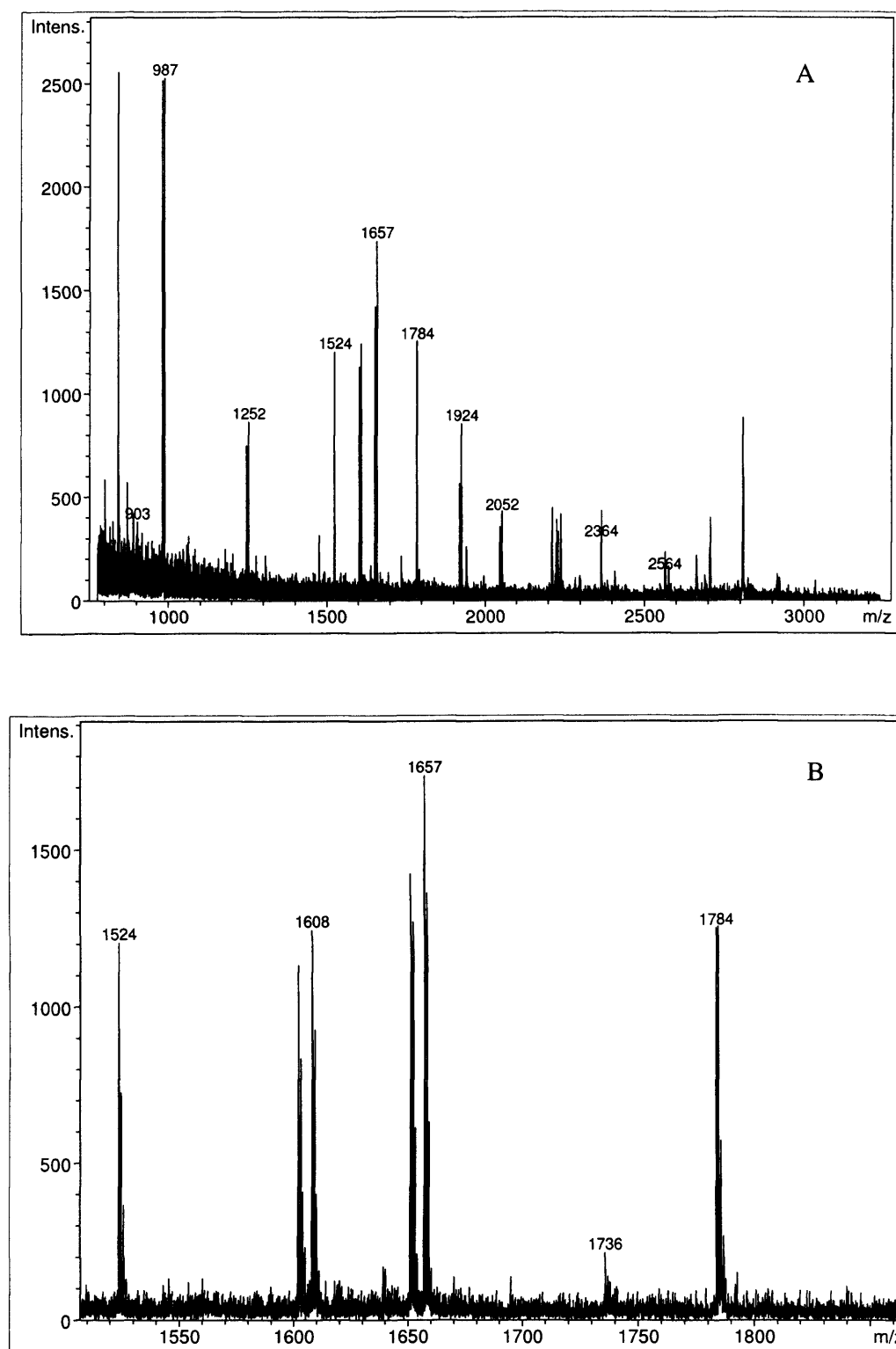


Figure 6-6 *RhoGDI mass spectrum.* MALDI-TOF spectrum from spot s2 (see Fig. 6-5). A full range spectrum (A); a close up of one area of interest (B). Duplicate peaks differ in mass by 6 Da, which corresponds to [^{13}C Arg], of the stimulated peptide variant. Observation of phosphorylated peptides is marginal (see Discussion for explanation).

Obs mass	Exp mass	Δ	Peptide
948.587	948.539	0.048	51-58
979.509	979.487	0.02	128-134
985.524	985.534	-0.010	128-134 + [^{13}C] Arg
1244.570	1244.549	0.021	142-152
1250.606	1250.596	0.01	142-152 + [^{13}C] Arg
1426.688	1426.523	0.165	142-152+ 2P + 1 Ox Met + [^{13}C] Arg
1600.766	1600.755	0.011	139-152
1606.784	1606.802	-0.08	139-152 + [^{13}C] Arg
1649.900	1649.909	-0.009	59-74
1655.919	1655.956	-0.037	59-74 + [^{13}C] Arg
1782.808	1782.729	0.079	139-152 + 2P + 1 Ox Met + [^{13}C] Arg
1782.808	1782.801	0.007	153-167
1916.929	1916.932	-0.03	34-49
1922.898	1922.979	-0.081	34-49 + [^{13}C] Arg
1985.956	1986.812	0.144	121-134+ 3P + 1 Ox Met + [^{13}C] Arg
2363.125	2363.106	0.019	181-199
2575.239	2575.308	-0.069	75-98

Table 6-1 *Table of RhoGDI peptides recovered from spot s2.* Masses observed from the spectrum correspond to the theoretical tryptic-digests of RhoGDI. Peptides are represented as amino acid numbers in the sequence of the protein, with observed modifications: [^{13}C] Arg-arginine residue labelled with ^{13}C ; Ox Met-oxidized methionine; P-phosphate group; Δ -the difference between the mass observed and the mass expected..

From spot s2 in total 17 peptides were identified, 14 of which trypsin had cleaved after Arg residue and 3 had Lys as the peptide-end residue. Out of the 14 residues with Arg-endings, 6 peptides were identified that belong to the unstimulated form of Rho GDI and 8 peptides that belong to the stimulated RhoGDI (they contained ^{13}C -Arg) (Table 6-1). Only 2 arginine-ending peptides (peptide 51-58 and peptide 121-134, Table 6-1) were not paired - their stimulated [^{13}C -Arg]/unstimulated [^{12}C -Arg] counterparts were not identified from the spectrum. 2 [^{13}C -Arg] peptides (142-152 and 139-152) were identified with two phosphorylations in the peptides. This is essentially the same peptide in its shorter and longer form. The unphosphorylated forms of these peptides were also identified, both as unstimulated [^{12}C -Arg] and stimulated [^{13}C -Arg] variants.

Peptide (121-134) was also observed as a [^{13}C -Arg] form with three phosphorylations, but unphosphorylated forms of this peptide were not observed.

Obs mass	Exp mass	Δ	Peptide
979.574	979.487	0.087	128-134
985.507	985.534	-0.027	128-134 + [^{13}C] Arg
1244.503	1244.549	-0.046	142-152
1250.531	1250.596	-0.065	142-152 + [^{13}C] Arg
1426.684	1426.523	0.161	142-152+ 2P + 1 Ox Met + [^{13}C] Arg
1600.687	1600.755	-0.068	139-152
1606.704	1606.802	0.087	139-152 + [^{13}C] Arg
1649.850	1649.909	-0.027	59-74
1655.877	1655.956	-0.046	59-74 + [^{13}C] Arg
1782.738	1782.729	0.009	139-152 + 2P + 1 Ox Met + [^{13}C] Arg
1782.738	1782.801	-0.063	153-167
1916.899	1916.932	0.087	34-49
1922.915	1922.979	-0.027	34-49 + [^{13}C] Arg
2363.084	2363.106	-0.046	181-199
2575.561	2575.308	0.253	75-98

Table 6-2 *Table of RhoGDI peptides recovered from spot s3.* Masses observed from the spectrum correspond to the theoretical tryptic-digests of RhoGDI. Peptides are represented as amino acid numbers in the sequence of the protein, with observed modifications: [^{13}C] Arg-arginine residue labelled with ^{13}C ; Ox Met- oxidized methionine; P-phosphate group; Δ -the difference between the mass observed and the mass expected.

Spot s3 had in total 15 peptides identified as RhoGDI peptides (Table 6-2), 3 with Lys residues and 12 as peptides with Arg at the end of the amino acid sequence. 5 [^{12}C -Arg]-peptides have their [^{13}C -Arg]-peptide pairs and 2 additional [^{13}C -Arg]-peptide pairs in their phosphorylated form (139-152 and 142-152). All of the identified peptides were also identified in spot s2.

From spot s4, 19 RhoGDI peptides were identified: 4 with Lys-ending and 15 with Arg ending (Table 6-3). In addition to the peptides identified in spots s2 and s3, 2 peptides

that were identified solely in s4 had phosphorylation sites. Peptide 187-199 observed with one phosphate group had a Lys at the end of the amino acid sequence, therefore it was not possible to distinguish whether this peptide belonged to the stimulated or unstimulated form of RhoGDI. Peptide 121-134, observed as a [^{13}C -Arg] peptide with 2 phosphate groups, was also identified in spot s2 where it was observed with 3 phosphate groups.

In all three RhoGDI spots the consistent change was the dually-phosphorylated peptide (139-152) $M+H^+ = 1782.7$ (Tables 6-1, 6-2 and 6-3 and Fig. 6-6) IDKTDYMVGSYGPR that appeared after treatment with ET. This peptide has four potential amino acid residues as candidates for phosphorylation: Thr¹⁴², Tyr¹⁴⁴, Ser¹⁴⁸ and Tyr¹⁴⁹.

The unphosphorylated form of the peptide was also observed in both unstimulated ($M+H^+ = 1600.7$) and stimulated ($M+H^+ = 1606.8$) RhoGDI. In addition to this the shorter version of the peptide (TDYMVGSYGPR) was present in the spectrum with all three peaks: $M+H^+ = 1244.5$ representing the unphosphorylated peptide obtained from unstimulated cells; $M+H^+ = 1250.6$ representing the unphosphorylated peptide obtained from stimulated cells; and $M+H^+ = 1426.5$ representing the dually phosphorylated RhoGDI peptide from stimulated cells.

The mass of $M+H^+ = 1782.8$ also covers another peptide from RhoGDI (153-167) (AEEYEFLTPMEEAPK) (Tables 6-1, 6-2 and 6-3). Both peptides (139-152 and 153-167) could therefore be giving rise to the peak with such high intensity (Fig. 6-6).

Obs mass	Exp mass	Δ	Peptide
948.508	948.539	-0.031	51-58
979.442	979.487	-0.045	128-134
985.461	985.534	-0.073	128-134 + [¹³ C] Arg
1244.462	1244.549	-0.087	142-152
1250.471	1250.596	-0.125	142-152 + [¹³ C] Arg
1426.396	1426.523	-0.127	142-152+ 2P + 1 Ox Met + [¹³ C] Arg
1600.641	1600.755	-0.114	139-152
1606.659	1606.802	-0.143	139-152 + [¹³ C] Arg
1641.699	1641.814	-0.115	187-199
1649.798	1649.909	-0.111	59-74
1655.817	1655.956	-0.139	59-74 + [¹³ C] Arg
1721.590	1721.781	0.191	187-199 + P
1782.685	1782.729	-0.044	139-152 + 2P + 1 Ox Met + [¹³ C] Arg
1782.685	1782.801	-0.116	153-167
1889.835	1889.850	-0.015	121-134+ 2P + [¹³ C] Arg
1916.861	1916.932	-0.071	34-49
1922.876	1922.979	-0.103	34-49 + [¹³ C] Arg
2363.163	2363.106	0.057	181-199
2575.329	2575.308	0.021	75-98

Table 6-3 *Table of RhoGDI peptides recovered from spot s4.* Masses observed from the spectrum correspond to the theoretical masses of tryptic-digests of RhoGDI. Peptides are represented as amino acid numbers in the sequence of the protein, with observed modifications: [¹³C] Arg-arginine residue labelled with ¹³C; Ox Met- oxidized methionine; P-phosphate group; Δ -the difference between the mass observed and the mass expected.

Mass observed	Mass expected	Obs.-Exp.	Ion
289.9	290.15	0.25	y5 ⁺²
346.1	346.17	0.07	a6-NH ₃ ⁺²
420.7	420.2	0.5	a7 ⁺²
492.4	492.26	-0.14	y4
502.9	503.24	-0.34	b9-H ₂ O ⁺²
620	619.8	0.2	y6-NH ₃
628.1	628.29	-0.19	b11-H ₂ O ⁺²
636.5	636.31	0.19	y6
637.6	637.29	0.31	b11 ⁺²
651.6	651.81	-0.21	a12 ⁺²
562	562.26	-0.26	y5-NH ₃
687.3	687.33	-0.03	y12 ⁺²
691.8	691.33	0.47	a6-NH ₃
705.6	705.32	0.28	b13-H ₂ O
708.6	708.36	0.24	a6
714.7	714.33	0.37	b13 ⁺²
717	717.37	-0.37	y7-H ₂ O
718.2	718.34	-0.14	b6-H ₂ O
719.6	719.33	0.28	b6-NH ₃
735.5	735.38	0.12	y7
736.4	736.35	0.15	b6
850.2	850.37	-0.17	b7-H ₂ O
938.9	938.47	0.43	a8
949.2	949.43	-0.13	b8-NH ₃
966.2	966.46	-0.26	b8
1012.3	1012.46	-0.16	y9-NH ₃
1023.7	1023.48	0.22	b9
1093.4	1094.43	-0.03	b10-NH ₃
1127.1	1127.48	-0.17	y10-NH ₃
1228.6	1228.53	0.43	y11-NH ₃

Table 6-4 *Table of masses observed for the ion 801⁺²*. Peptide observed in the MALDI-TOF spectrum of trypsin-digested RhoGDI, m/z 1600, was sequenced by nanospray MS/MS. The masses observed in the spectrum are shown with their deviation from theoretical values. 'b', 'y' and 'a' ions shown are most useful for sequencing and were used to determine the sequence of the peptide illustrated in Fig. 6-7.

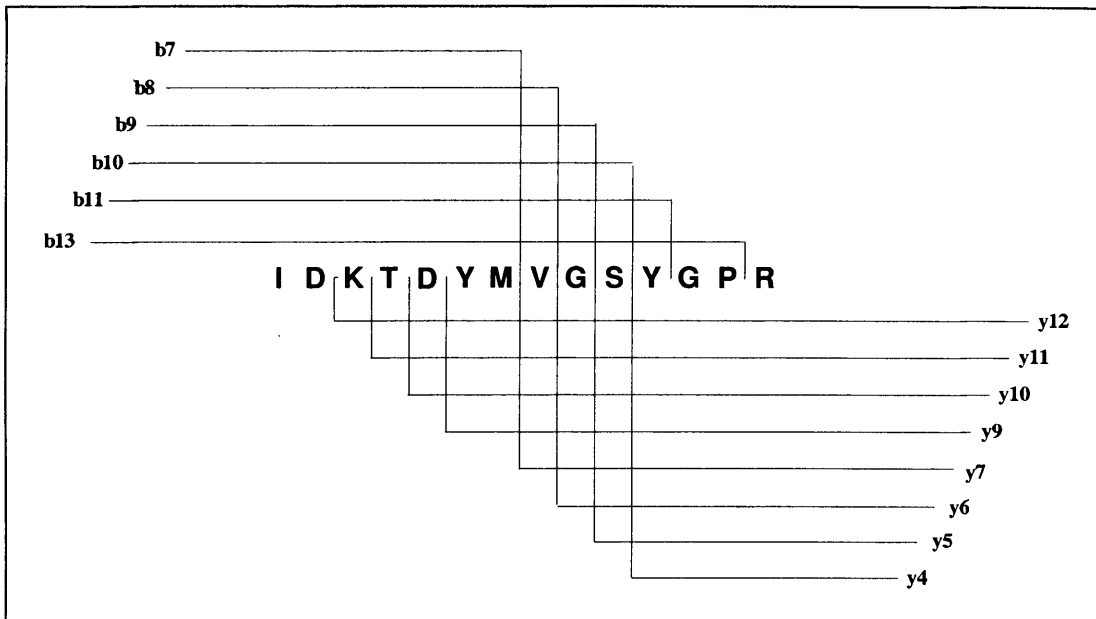


Figure 6-7 *Sequence analysis of ion 801⁺² by MS/MS analysis.* The unstimulated RhoGDI peptide $m+H^+ = 1600$, identified in MALDI-TOF analysis, was sequenced by nanospray ion trap mass spectrometry. The figure illustrates the sequence generated from the ions recovered from the spectrum.

Mass observed	Mass expected	Obs.-Exp.	Ion
287.3	287.15	0.15	b5 ⁺²
357.4	357.21	0.2	b3
360.1	360.21	-0.11	b6-NH ₃ ⁺²
413.2	413.24	-0.04	a4-NH ₃
441.0	441.23	-0.23	b4-NH ₃
461	461.22	-0.22	a8-NH ₃ ⁺²
469.9	469.74	0.16	a8 ⁺²
474.1	474.73	-0.63	b8-H ₂ O ⁺²
483.4	483.7	-0.3	b8 ⁺²
489.2	489.73	-0.53	a9-NH ₃ ⁺²
503.4	503.24	0.16	b9-H ₂ O ⁺²
512.1	512.24	-0.14	b9 ⁺²
528.4	528.27	0.13	a5-NH ₃
533.1	533.25	-0.15	a10-NH ₃ ⁺²
545.3	545.29	0.01	a5
546.2	546.76	-0.56	b10-H ₂ O ⁺²
555.8	555.76	0.04	b10+2
556.6	556.26	0.34	b5-NH ₃
573.3	573.29	0.01	b5
623.3	623.4	-0.1	a11 ⁺²
651.81	651.8	0.01	a12 ⁺²
656.7	656.8	-0.1	b12-H ₂ O ⁺²
657.4	657.29	0.11	b12-NH ₃ ⁺²
665.7	665.8	-0.1	b12 ⁺²
674.7	674.8	-0.1	b12+H ₂ O ⁺²
691.2	691.33	-0.13	a6-NH ₃
691.7	691.82	-0.12	a13-NH ₃ ⁺²
700.8	700.33	0.47	a13 ⁺²
705.2	705.32	-0.12	b13-H ₂ O ⁺²
708.9	708.36	0.54	a6
714.6	714.33	0.27	b13 ⁺²
718.3	718.34	-0.04	b6-H ₂ O
719.6	719.33	0.27	b6-NH ₃
723.4	723.34	0.06	b13+H ₂ O ⁺²
736.5	736.35	0.15	b6
839.9	839.4	0.5	a7
850.4	850.37	0.03	b7-NH ₃
867.7	867.39	0.31	b7
948.4	948.45	-0.05	b8- H ₂ O
966.6	966.48	0.12	b8
978.1	978.46	-0.36	a9- NH ₃
1005	1005.47	-0.47	b9- H ₂ O

Table 6-5 *Table of masses observed for the ion 804⁺².* (Continued overleaf)

Mass observed	Mass expected	Obs.-Exp.	Ion
1024.4	1023.48	-0.08	b9
1082.5	1082.52	-0.02	a10
1110	1110.5	-0.5	b10
1255.4	1255.57	-0.13	b11- H ₂ O
1256.4	1256.55	-0.15	b11- NH ₃
1273.5	1273.58	-0.08	b11
1302.5	1302.6	-0.1	a12
1313.1	1313.57	-0.47	b12- NH ₃
1330.8	1330.6	0.2	b12
1400.1	1399.6	0.5	a13
585.9	585.29	0.61	y5
642.2	642.31	-0.11	y6
741.1	741.38	-0.28	y7
1251.7	1251.56	0.14	y11
252.7	252.63	0.07	y4 ⁺²
521.1	521.25	-0.15	y9 ⁺²
750.9	750.84	0.06	y13 ⁺²
578.7	578.76	0.04	y10 ⁺²
693.1	693.33	-0.23	y12 ⁺²

Table 6-5 (cont'd) *Table of masses observed for the ion 804⁺².* Peptide observed in the MALDI-TOF spectrum of trypsin-digested RhoGDI, m/z 1606, was sequenced by nanospray MS/MS. The masses observed in the spectrum are shown with their deviation from theoretical values. 'b', 'y' and 'a' ions shown are the most useful for sequencing and were used to determine the sequence of the peptide illustrated in Fig. 6-8.

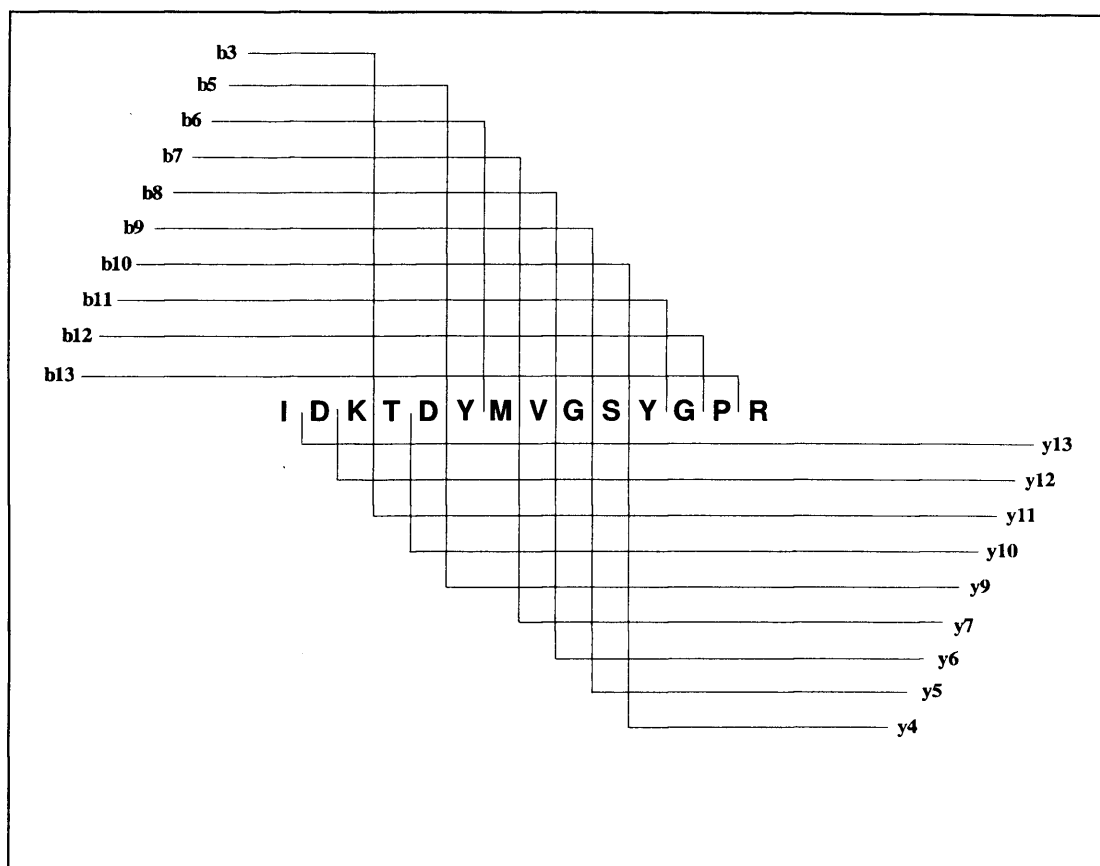


Figure 6-8 *Sequence analysis of the ion 804⁺² by MS/MS analysis.* The stimulated RhoGDI peptide $m+H^+ = 1606$, identified in MALDI-TOF analysis, was sequenced by nanospray ion trap mass spectrometry. The figure illustrates the sequence generated from the ions recovered from the spectrum.

In order to confirm that the peptide (139-152) in its dually phosphorylated stimulated form is below the peak $M+H^+ = 1782.8$, the peptide was sequenced using a nanospray ion trap mass spectrometer (Table 6-6 and Figs. 6-9 and 6-10). The spectrum obtained from MS/MS of the peak at $m/z = 892^{+2}$, corresponding to $M+H^+ = 1782.8$ is given in Fig. 6-9. y, b and a ions are labelled. These ions correspond to the masses of pieces of peptide sequence which include either the N-terminus (a, b) or the C-terminus (y). These ions are therefore the most useful for sequencing peptides, as compared to internal fragment ions, which can come from anywhere in the peptide and do not include either terminus. In addition to the y, a and b ions, many internal fragments were also identified, but these were not included in the table. For the complete list of ions used in the identification of the sequence see Table 6-6.

The fragmentation spectrum shown in Fig. 6-9 contains ion signals corresponding to the neutral loss of one ($m/z = 843.4$, $((1782.8 + 2H^+ - 98)/2)$) and two ($m/z = 794.4$, $((1782.8 + 2H^+ - 98*2)/2)$) molecules of phosphoric acid (H_3PO_4 , 98 Da) from the parent ion. These signals indicate the presence of two phosphorylated residues in the peptide. From the series of b and y fragment ions (indicated in Table 6-6) the sequence of the peptide was revealed (Fig 6-10a) with Tyr¹⁴⁴ and Ser¹⁴⁸ as the two phosphorylation sites.

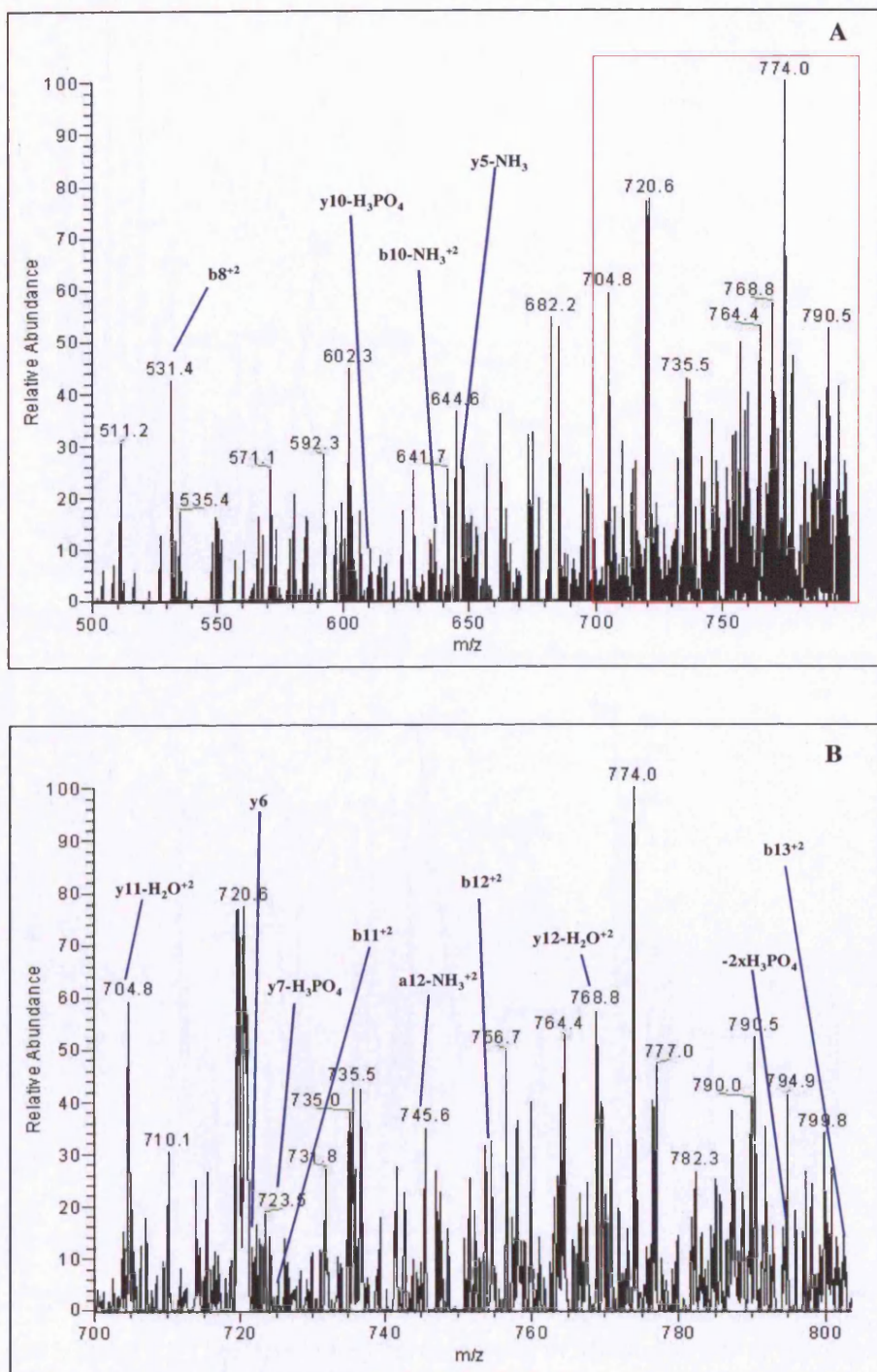


Figure 6-9 The spectrum obtained from MS/MS analysis of the peak $m/z = 892^{+2}$ ($M+H^+$ = 1782). (Continued overleaf)

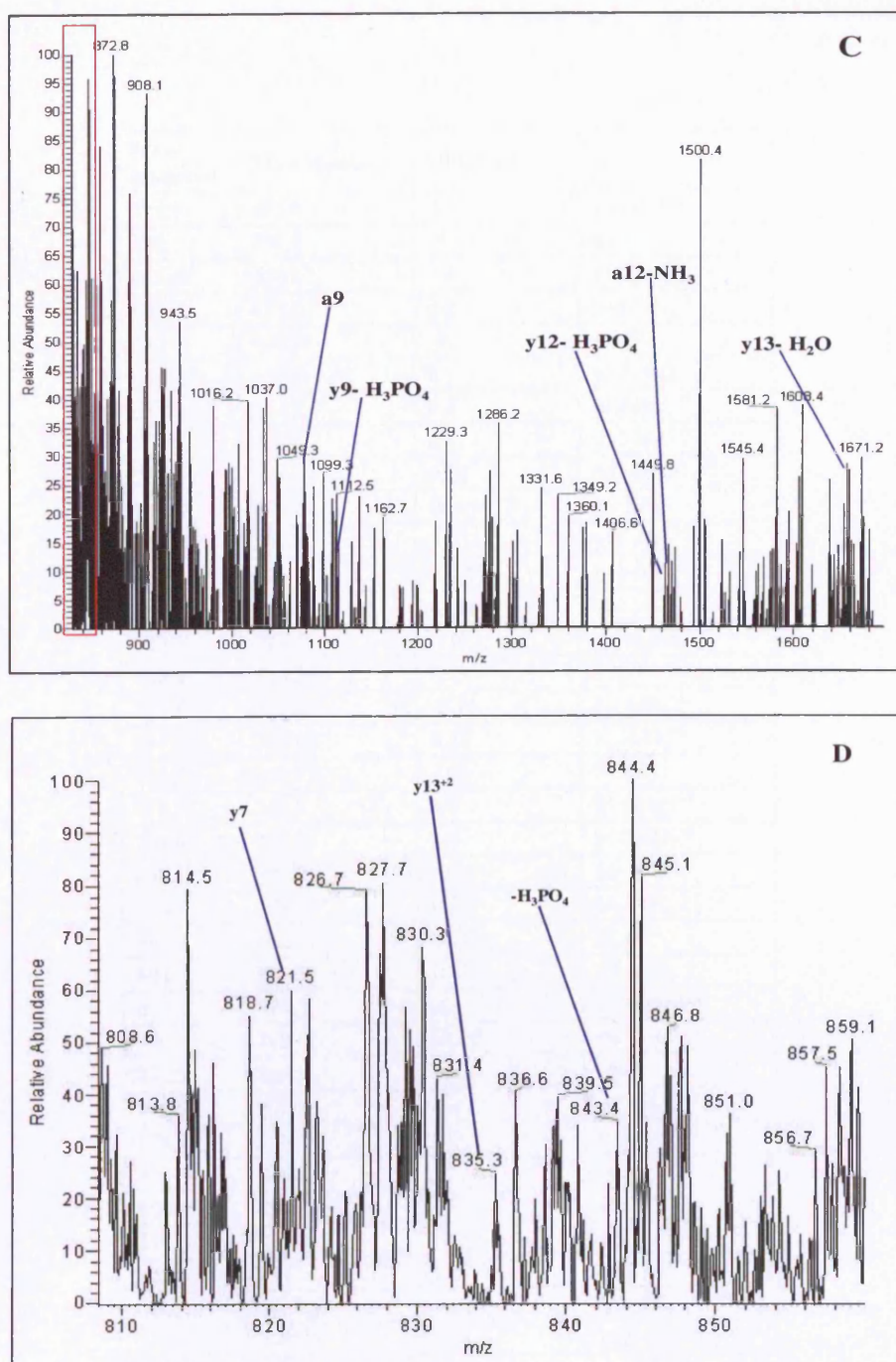


Figure 6-9 (cont'd) The spectrum obtained from MS/MS analysis of the peak $m/z = 892^{+2}$ ($M+H^+ = 1782$). The ions identified in the spectrum and the sequence generated from them are represented in Table 6-6 and Fig. 6-10. B and D show details from the dense section in A and C (red rectangle). Backbone sequence ions (y, a, b) are labelled above the spectra. Ion signals obtained from the neutral loss of one ($m/z = 843.4$) and two ($m/z = 794.4$) molecules of phosphoric acid are indicated. MALDI data on RhoGDI phosphorylation were marginal (Fig 6-6) (see Discussion for explanation).

Mass observed	Mass expected	Obs.-Exp.	Ion
474.9	475.6	0.7	y8-NH ₃ ⁺²
531.4	531.7	-0.3	b8 ⁺²
613.8	614.2	0.4	y10-H ₃ PO ₄ ⁺²
635.4	635.2	0.2	b10-NH ₃ ⁺²
647.9	648.23	-0.3	y5-NH ₃
654.2	654.9	0.7	y10-H ₂ O ⁺²
654.9	654.7	0.2	y10-NH ₃ ⁺²
704.7	704.8	0.1	y11-H ₂ O ⁺²
704.8	704.78	0.02	b12-H ₃ PO ₄ ⁺²
704.8	705.2	0.4	y6-NH ₃
705.2	704.8	0.5	y11-NH ₃ ⁺²
716.5	716.75	-0.25	b11-NH ₃ ⁺²
722.1	722.28	-0.18	y6
723.5	723.37	0.13	y7-H ₃ PO ₄
725.3	725.26	0.04	b11 ⁺²
731.1	731.26	-0.16	a ₁₂ -NH ₃ ⁺²
745.6	745.25	0.35	b ₁₂ -NH ₃ ⁺²
753.8	753.77	0.03	b12 ⁺²
768.7	768.8	0.1	y12-H ₂ O ⁺²
768.8	769.2	0.4	y12-NH ₃ ⁺²
798	798.31	-0.31	b6-H ₂ O
799.8	799.29	0.51	b6- NH ₃
802.4	802.29	0.11	b13 ⁺²
804.7	804.32	0.38	y7- NH ₃
811.6	811.3	0.3	b13+H ₂ O ⁺²
816.3	816.32	-0.02	b6
821.5	821.35	0.15	y7
826.7	826.3	0.4	y13-H ₂ O ⁺²
826.8	826.7	0.1	y13-NH ₃ ⁺²
835.3	835.3	0	y13 ⁺²
1090.9	1091.45	-0.55	a9
1113.1	1113.43	-0.33	y9- H ₃ PO ₄
1410.5	1411.1	0.5	y11-NH ₃
1432.1	1432.48	-0.38	b ₁₁ -NH ₃
1457.7	1457.6	0.1	y12- H ₃ PO ₄
1461.6	1461.51	0.09	a ₁₂ -NH ₃
1488.7	1488.52	0.18	b12-H ₂ O
1524.4	1524.54	-0.14	b12+H ₂ O
1572.4	1572.63	-0.23	y13- H ₃ PO ₄
1652.6	1652.6	0.00	y13- H ₂ O

Table 6-6 *Table of masses observed for the ion 892⁺².* The peptide observed in the MALDI-TOF spectrum of trypsin-digested RhoGDI, m/z 1782, was subjected to nanospray sequencing. The masses observed in the spectrum are shown with their deviation from theoretical values. 'b', 'y' and 'a' ions shown are the most useful for sequencing and were used to determine the sequence of the peptide illustrated in Fig. 6-10.

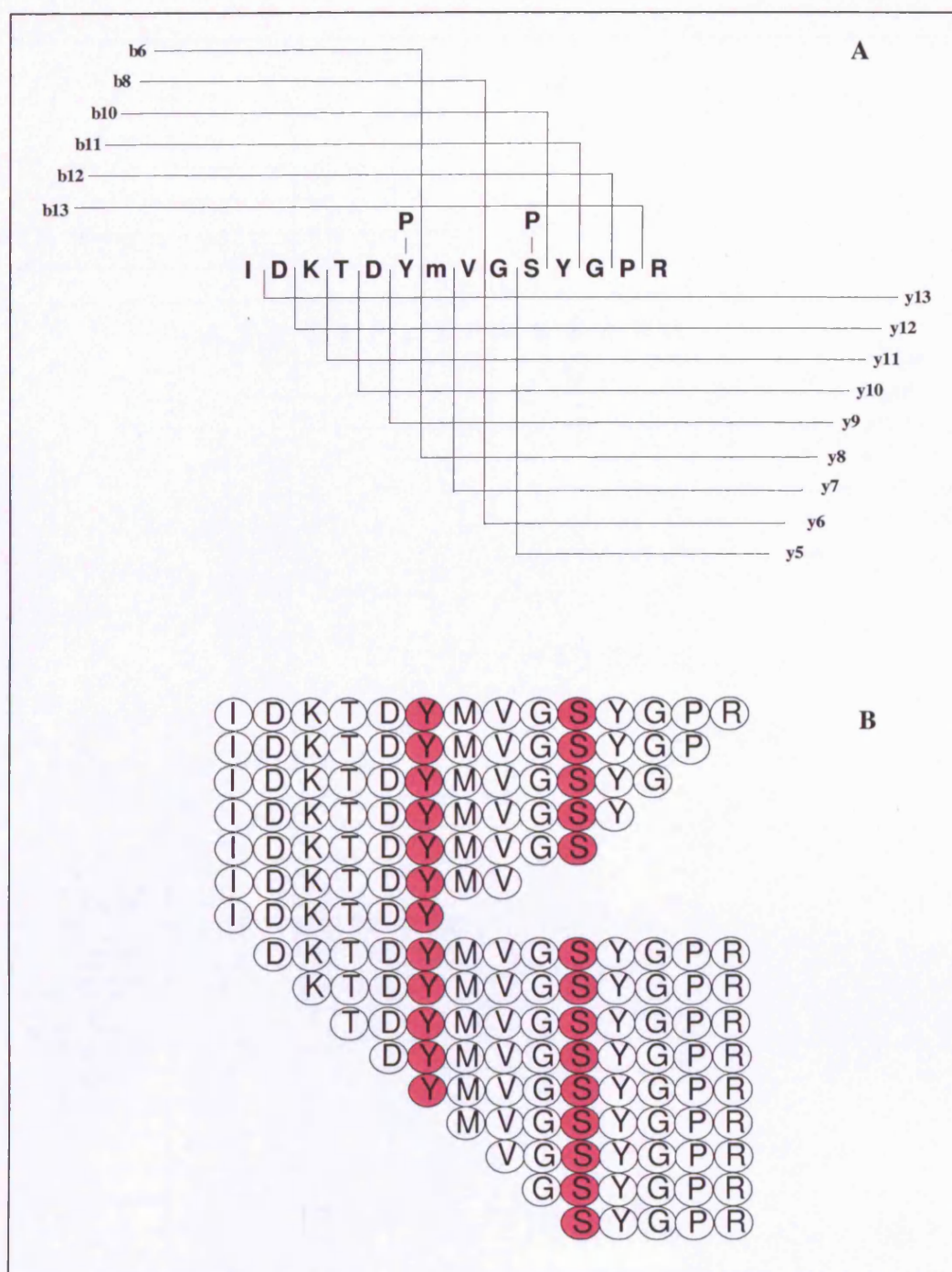


Figure 6-10 Sequence analysis of the ion 892⁺² by MS/MS analysis. (Continued overleaf)

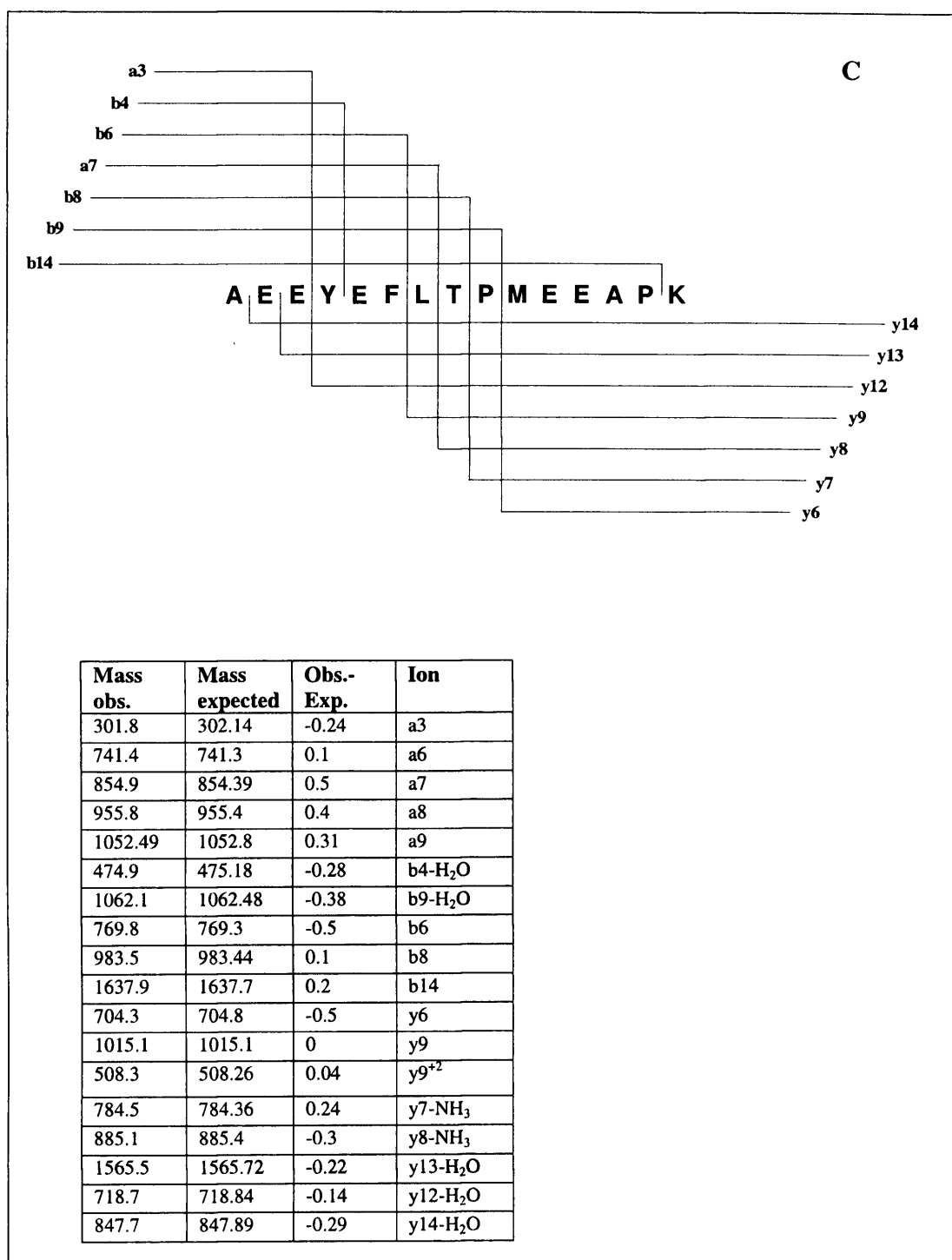


Figure 6-10 (cont'd) *Sequence analysis of the ion 892⁺² by MS/MS analysis.* (A) The stimulated RhoGDI peptide $m+H^+ = 1782$, identified in MALDI-TOF analysis, was sequenced by nanospray ion trap mass spectrometry. The figure illustrates the sequence generated from the ions recovered from the spectrum, 'P' represents a phosphate group. (B) A schematic representation of the ions generated by collision-induced dissociation (CID). The coloured circles represent phosphorylated Ser/Tyr residues. (C) Fragment ions obtained by MS/MS of the ion 892²⁺ that correspond to the RhoGDI peptide 153-167.

```

GDIR_MOUSE    MAEQEPTAEQLAQIAAENEDEHSVNYKPPAQKSIQEIQELDKDDESLRKYKEALLGRVA 60
GDIR_RAT      MAEQEPTAEQLAQIAAENEDEHSVNYKPPAQKSIQEIQELDKDDESLRKYKEALLGRVA 60
               *****

GDIR_MOUSE    VSADPNVPNVIVTRLTLVCSTAPGPLELDLTGDLESFKKQSFVLKEGVEYRIKISFRVNR 120
GDIR_RAT      VSADPNVPNVIVTRLTLVCSTAPGPLELDLTGDLESFKKQSFVLKEGVEYRIKISFRVNR 120
               *****

GDIR_MOUSE    EIVSGMKYIQHTYRKGVKIDKTDYMGVSYGPRAEYEFLTPMEEAPKGMLARGSYNIKS 180
GDIR_RAT      EIVSGMKYIQHTYRKGVKIDKTDYMGVSYGPRAEYEFLTPMEEAPKGMLARGSYNIKS 180
               *****

GDIR_MOUSE    FTDDDKTDHLSWEWNLTIKKEWKD 204
GDIR_RAT      FTDDDKTDHLSWEWNLTIKKEWKD 204
               *****

```

Figure 6-11 *Comparison of rat and mouse RhoGDI.* Sequences were aligned with ClustalW. The peptide identified with mass spectrometry is labelled in red with phosphorylated residues in blue. Sequence patterns recognized usually by Ser/Thr kinases are highlighted in light-blue. The consensus sequence is labelled with stars. Gi numbers are: mouse - RhoGDI-1, 31982030; rat - similar to RhoGDI-1, 34875656.

```

GDI-S2        MAEQEPTAEQLAQIAAENEDEHSVNYKPPAQKSIQEIQELDKDDESLRKYKEALLGRVA 60
GDI-S3        MAEQEPTAEQLAQIAAENEDEHSVNYKPPAQKSIQEIQELDKDDESLRKYKEALLGRVA 60
GDI-S4        MAEQEPTAEQLAQIAAENEDEHSVNYKPPAQKSIQEIQELDKDDESLRKYKEALLGRVA 60

GDI-S2        VSADPNVPNVIVTRLTLVCSTAPGPLELDLTGDLESFKKQSFVLKEGVEYRIKISFRVNR 120
GDI-S3        VSADPNVPNVIVTRLTLVCSTAPGPLELDLTGDLESFKKQSFVLKEGVEYRIKISFRVNR 120
GDI-S4        VSADPNVPNVIVTRLTLVCSTAPGPLELDLTGDLESFKKQSFVLKEGVEYRIKISFRVNR 120

GDI-S2        EIVSGMKYIQHTYRKGVKIDKTDYMGVSYGPRAEYEFLTPMEEAPKGMLARGSYNIKS 180
GDI-S3        EIVSGMKYIQHTYRKGVKIDKTDYMGVSYGPRAEYEFLTPMEEAPKGMLARGSYNIKS 180
GDI-S4        EIVSGMKYIQHTYRKGVKIDKTDYMGVSYGPRAEYEFLTPMEEAPKGMLARGSYNIKS 180

GDI-S2        FTDDDKTDHLSWEWNLTIKKEWKD 204
GDI-S3        FTDDDKTDHLSWEWNLTIKKEWKD 204
GDI-S4        FTDDDKTDHLSWEWNLTIKKEWKD 204

```

Figure 6-12 *Differences in RhoGDI isoelectric isoforms.* The peptides identified with mass spectrometry in three different isoelectric (phospho)isoforms (see Fig. 6-5 and Tables 6-1, 6-2 and 6-3) are labelled in yellow. Residues labelled in red are phosphorylated in stimulated RhoGDI. Residues labelled in light blue are also potentially phosphorylated in stimulated RhoGDI.

6.3 Discussion

The results from the previous chapter showed that RhoGDI is purified in the enriched phosphoprotein fraction in rat fibroblasts, and that the phosphorylation level of this protein increased 4 mins after cells were stimulated with ET-1. In the experiments described in this chapter the goal was to further examine the phosphorylation changes of RhoGDI induced by ET-1. The results reported in this section indicate the identity of the RhoGDI peptide that is phosphorylated after ET-treatment. In addition, the sequencing of this peptide revealed previously unknown sites of *in vivo* phosphorylation of RhoGDI.

Initially immunoprecipitated RhoGDI was resolved on a SDS-PAGE gel (Fig. 6-2). Successful immunoprecipitation of RhoGDI was verified. In addition a protein band (Mw~80kDa) was observed to co-precipitate with RhoGDI after ET-1 stimulation. The efforts to identify this protein during the course of this PhD project were unsuccessful due to the following reasons:

- the separation of the proteins in SDS-PAGE was not good enough to identify the protein with MALDI-TOF MS; and
- the protein was not observed in 2D gels of the same experiment. The pI value of the protein may have been outside the pH strip range used for the first dimension (pH 4-7 and pH 3-10 strips were tried).

The phosphorylation of RhoGDI was immunodetected with phospho-(Ser) PKC substrate antibody (Fig. 6-2 B). This antibody detects proteins phosphorylated at serine, surrounded by arginine or lysine in -2 and +2 positions and a hydrophobic amino acid at the +1 position. RhoGDI was phosphorylated in unstimulated as well as ET-1

stimulated cells. In chapter 5 phosphorylation of RhoGDI was also observed in unstimulated cells (Fig. 5-1). However, the level of phosphorylation of RhoGDI (observed with phospho-(Ser) PKC substrate antibody) after ET-1 stimulation did not change significantly. RhoGDI could have multiple phosphorylation sites and not all of them modified by stimulation in the same way. There are four possible Ser residues in RhoGDI that fit in the sequence motif recognized by the anti-phospho PKC substrate antibody: Ser⁴⁷, Ser⁹⁶, Ser¹⁰¹ and Ser¹¹⁵ (see Fig. 6-9 for location).

The unknown protein co-precipitating with RhoGDI was also detected with this antibody suggesting that it is phosphorylated on Ser. From the results obtained, it can not be ascertained whether this protein is phosphorylated as a result of ET-1 stimulation. The stronger signal which was observed could come from the higher abundance of this protein in the stimulated cell extract (Fig. 6-2 A).

Incidentally the immunoprecipitation experiments revealed that ET-1 induces actin reorganization/polymerization in NRK-49F cells (Fig. 6-3). Namely, actin was binding with high affinity to sepharose and was purified in the control for non-specific binding. ET-1 was previously reported to induce actin reorganization and stress fibre formation in different cells, for example:

- ET-1 induced actin polymerization in airway smooth muscle cells and this process involved the activation of Rho (Hirshman and Emala, 1999); and
- in astrocytes ET-1 stimulated FAK/Src pathway activation and the formation of stress fibres (Cazaubon *et al.*, 1997).

The phosphorylation of actin observed in chapter 5 may regulate the reorganization of actin observed here. In NRK-49F cells ET-1 induces anchorage-independent growth

and on the other hand actin polymerization is important for cell adhesion (Lahaye *et al.*, 1999; Cazaubon *et al.*, 1997). In this view it would be interesting to define the kind of cytoskeletal reorganization that ET-1 induces and how that is related to anchorage-independent cell growth.

2D gel separation of the immunoprecipitated RhoGDI revealed an array of spots forming a train-like pattern in the region corresponding to RhoGDI (Fig. 6-5). This pattern stems from the shift in pI of the protein, indicating the existence of additional charge. Additional charge is often due to protein phosphorylation (Smith *et al.*, 1984; Choudhary *et al.*, 2000) which adds negative charge to the net protein charge rendering the molecule movement closer to the acidic end. Additional charge can also be attributed to protein glycosylation but this is usually accompanied by an increase in molecular weight, so it was not considered as a possibility in the situation presented in Fig. 6-5. The results therefore suggest that RhoGDI is resolved on a 2D gel in several differentially phosphorylated forms. The most acidic isoelectric form (pH~5.0) of RhoGDI was predominant (s4, Fig. 6-5), and this one is likely to have the highest proportion of phosphorylation. Based on relative spot-intensity comparisons, the order of relative abundance of RhoGDI isoelectric isoforms is : s4>s2>s3. It could be speculated that s2 and s4 are major isoforms and s3 is a transient one, which the protein assumes in between the other two isoforms. There is an additional spot at pH~4.9 more acidic than s4, highly likely to be another GDI isoform, which was not identified in the course of these experiments.

All three spots were identified by peptide mass fingerprinting to be RhoGDI-1 (gil31982030) from the house mouse. Fibroblasts used for the studies were from rat but the fact that the top score in database searches was a mouse form of GDI protein is not

significant as this protein is identical in sequence to its rat homologue, similar to RhoGDI-1 (gil34875656) (Fig. 6-11). In addition to this the molecular weight of the identified protein corresponds to the Mw of non-truncated RhoGDI-1, confirming that we are not looking at a different splice-variant. The protein sequence of rat homologue of GDI-1 was generated by computational translation of a genomic sequence and is supported by mRNA and EST evidence.

MALDI-TOF analysis of the three protein spots demonstrated that the majority of peaks identified were the same, but there were some peaks that were different among the spectra of the three different RhoGDI forms. Some of the different peaks were identified as phosphorylated peptides, agreeing with the observation of the pattern the protein forms on a 2D gel (Tables 6-1, 6-2 and 6-3). The most acidic spot s4 was shown to be the one with the most phosphorylation sites. The second most phosphorylated form is spot s2, which out of the three identified RhoGDI spots, was the most basic spot. The explanation of this result lies in the fact that phosphopeptides often give low-intensity signals or even no signals in MALDI-TOF spectra (Liao *et al.*, 1994), therefore spot s3 may have additional phosphorylation that was not detected. Additional experiments with the detailed sequence analysis of all potential RhoGDI phosphoisoforms are needed to evaluate the results observed in the experiments presented in this chapter.

SILAC methodology enabled the comparison of the unstimulated (^{12}C -Arg containing peptides) and stimulated (^{13}C -Arg containing peptides) RhoGDI in the same mass spectrum. The doublet peak-pair is the main feature of SILAC generated mass spectra of protein digests (Figs. 6-4 and 6-6). It consists of the first peak being the peptide that comes from the unstimulated cell pool (with normal Arg), and the second peak (6 Da to

the right of the first peak) which comes from the $^{13}\text{C6}$ -Arg labelled stimulated cell pool. Because protein extracts are mixed in equal proportion at the beginning of the experiment, the difference in peak intensities would correspond to the quantitative difference in the abundance of peptide from the unstimulated cell pool compared to the abundance of the same peptide in the stimulated cell pool.

A dually phosphorylated IDKTDYMGVGSYGPR peptide (139-152) was observed to be consistently phosphorylated after treatment with ET in mass spectra of all RhoGDI spots (Tables 6-1, 6-2 and 6-3). The mass of this peptide coincides with the mass of the peptide AEEYEFLTPMEEAPK (153-167), also observed in all mass spectra. Both peptides could be present in the trypsin digest and give rise to the high intensity signal at $M+H^+ = 1782.8$. Observation of the short form of peptide TDYMGVGSYGPR (142-152) again as a dually phosphorylated peptide ($M+H^+ = 1426.3$) in stimulated RhoGDI gave a more clear indication that the dual phosphorylation of this peptide is indeed present in the stimulated RhoGDI. In addition to the novel phosphorylation observed in the stimulated RhoGDI, unphosphorylated forms of the peptide were also identified through analysing spectra of both unstimulated ($M+H^+ = 1244.4$) and stimulated ($M+H^+ = 1250.4$) RhoGDI. Quantification of phosphopeptides is difficult in MALDI-TOF MS because the energies applied often cause dephosphorylation and this process is not quantitative which enables the detection of the same peptide in phosphorylated and unphosphorylated forms in the same spectrum (Liao *et al.*, 1994).

Therefore the observed unphosphorylated IDKTDYMGVGSYGPR peptide obtained from the stimulated cell pool could in fact have been dephosphorylated during spectrum acquisition. Because quantification of phosphorylation was not possible, the reproducibility of the spectra and the presence of all but one species of a single peptide

(unphosphorylated-unstimulated, phosphorylated-unstimulated, unphosphorylated-stimulated and phosphorylated-stimulated) were the criteria for the selection of peaks for electrospray analysis.

MALDI-TOF analysis just partially helped in evaluating potential RhoGDI peptides that undergo phosphorylation after ET-1 stimulation. A much clearer picture would be formed if the phosphorylated peptides of RhoGDI were enriched prior to MS analysis.

It should be noted here that the observation of phosphorylated peptides in MALDI-TOF MS is difficult and the peptides observed in this chapter (Fig. 6-1) were of very low intensity or masked by a high signal from a different unphosphorylated peptide. This could also explain a dense peak MS/MS spectrum (Fig. 6-9). The data based on shown spectra should therefore be taken with caution. However, repeated MS/MS analysis of RhoGDI from several different experiments in our group confirmed the pattern of phosphorylation observed in this chapter.

The peptide IDKTDYMGVGSYGPR has four potential phosphorylation sites, but only two of them have the very high probability score for phosphorylation: Tyr¹⁴⁴ and Ser¹⁴⁸ based on database predictions (Scansite at <http://scansite.mit.edu/> and Phosphobase at <http://www.cbs.dtu.dk/databases/PhosphoBase/>). In order to confirm the existence and establish the phosphorylation sites of this peptide it was sequenced with nanospray MS/MS. The results of the sequencing confirmed that this peptide is present in its dually phosphorylated form in the tryptic digest of ET-stimulated RhoGDI. Sequencing also revealed Tyr¹⁴⁴ and Ser¹⁴⁸ as the sites of modification. In the literature there are no reports on the phosphorylation of RhoGDI in fibroblasts. The phosphorylation of Thr residues of Ly-GDI, a hematopoietic cell homologue of RhoGDI-2, is the sole work

reported on phosphorylation sites of RhoGDI (Scherle *et al.*, 1993). In addition to this RhoGDI was shown to be a substrate for PKC α in response to thrombin induced activation of endothelial cells (Mehta *et al.*, 2001).

Phosphorylation of a Ser and a Tyr residue in the same molecule suggests participation of at least two kinases in this process. It is not uncommon that a protein is regulated by dual phosphorylation, examples being ERK1/2 and Gab2 (Her *et al.*, 1993; Momose *et al.*, 2003). Three families of protein kinases, namely the Ser/Thr kinases, the Tyr kinases and the dual specificity Ser/Thr/Tyr kinases are contenders for the regulation of RhoGDI function. On the other hand, negative regulation of GDI phosphorylation would be controlled by the activity of different phosphatases. Increasing evidence of RhoGDI-1 being found in various multi-protein signalling complexes (Tolias *et al.*, 1998; Hirao *et al.*, 1996; Abo *et al.*, 1994) support the view of a complex regulation of RhoGDI function.

The amino acid residues the phosphorylation of which was discovered in these studies do not fall within a consensus sequence for common kinases, although the Ser¹⁴⁸ is within the binding motif for 3-phosphoinositide dependent protein kinase-1 (PDK1). This is a Ser/Thr protein kinase that translocates from the cytosol to the membrane after stimulation (Anderson *et al.*, 1998), and this process could potentially closely follow the process of translocation of Rho to the membrane and its dissociation from RhoGDI. Substrates of PDK1 include fundamental signalling proteins: PKB, PKA, PKC zeta and ribosomal S6 kinase (Biondi, 2004). These kinases are also known to take part in ET-induced signalling pathways.

A comparison of the sequence of different RhoGDI proteins (Fig. 6-13) shows that the motif is evolutionarily conserved among different species and also between different GDI isoforms, with Ser¹⁴⁸ being entirely conserved from yeast to human, while Tyr¹⁴⁴ is present only in mammalian RhoGDI-1 and in human RhoGDI-3. In other isoforms of RhoGDI, Tyr is substituted by Phe (with the exception of yeast RhoGDI-1), an aromatic amino acid of the same size and hydrophobicity. It is also interesting to note that this segment of RhoGDI sequence forms the loop connecting β strands β 6 and β 7, which would suggest that it is more likely to evolve. The high level of conservation of this particular portion of the protein indicates that it may have an important function.

GDI-1_MOUSE	IDKTDYMVGSYGPRAE	154
GDI-1_HUMAN	IDKTDYMVGSYGPRAE	154
GDI-1_BOVIN	IDKTDYMVGSYGPRAE	154
GDI-2_MOUSE	VDKATFMVGSYGPRPE	150
GDI-2_HUMAN	VDKATFMVGSYGPRPE	151
GDI-2_BOVIN	VDKATFMVGSYGPRPE	150
GDI-3_MOUSE	VDKAIFMVGSYGPRAQ	175
GDI-3_HUMAN	VDKTVYMVGSYGPSAQ	175
GDI-1_CAVPO	IDKTDYMVGSYGPRAE	73
GDI-1_CAEEL	VENEKYMMGSYAPKLE	144
GDI-1_YEAST	VDKIDDHLGSYAPNTK	152
	::: :***.* :	

Figure 6-13 *Evolutionary conservation of IDKTDYMVGSYGPRAE motif in RhoGDI proteins.* Part of the sequence alignment, containing the peptide of interest, between different species is illustrated. Residues identical for all RhoGDI proteins are indicated by asterisk and conservative amino acid substitutions are identified by dots. CAVPO stands for Guinea pig, CAEEL for *Caenorhabditis elegans*. The GI numbers are: mouse GDI-1, 31982030; human GDI-1, 1707892; bovine GDI-1, 121107; mouse GDI-2, 2494703; human GDI-2, 1707893; bovine GDI-2, 13626951; mouse GDI-3, 2494704; human GDI-3, 4502225; guinea pig GDI-1, 21759490; *Caenorhabditis elegans* GDI-1, 2494705; yeast GDI-1 2494706.

The replacement of Tyr residue in RhoGDI isoforms other than RhoGDI-1, may account for the differential regulation these proteins have. GDI-1 is able to bind several Rho/Rac GTPases: RhoA, RhoB, Rac1, Rac2 and Cdc42; GDI-3 binds to RhoB whilst the GDI-2 Rho partner is still unidentified (Olofsson, 1999). In addition, GDI-3 is speculated not to be a cytosolic protein (Zalcman *et al.*, 1996) perhaps implying a different role compared to the other RhoGDI isoforms.

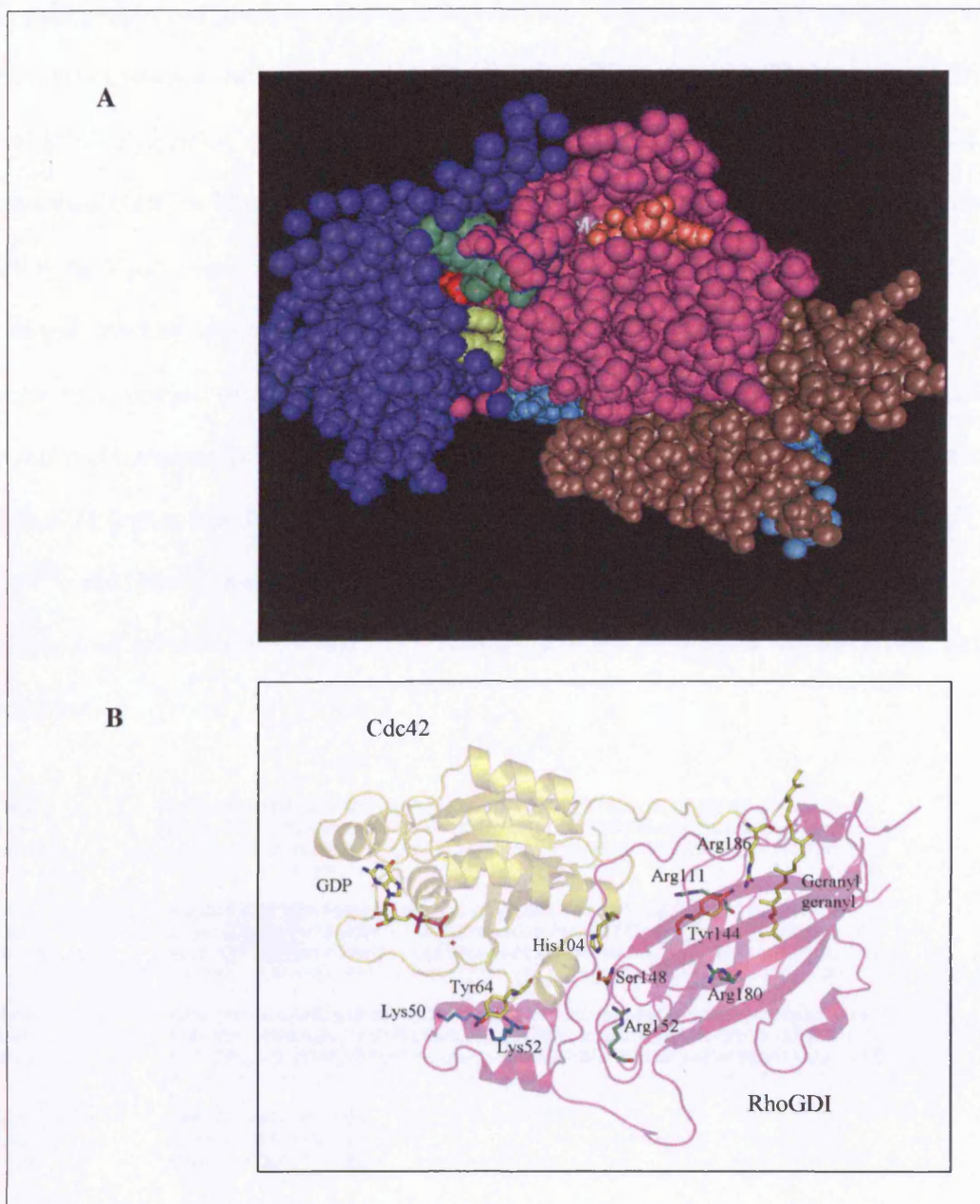


Figure 6-14 *Structure of the RhoGDI in complex with small GTPases.* (A) Space-fill representation of the human RhoA-RhoGDI complex. RhoGDI is in blue, RhoA is in purple, with the GDP molecule in brick. RhoGDI Ser¹⁴⁸, phosphorylated after ET-1 stimulation, is in red. YDRLRPL (amino acids 64-70) of RhoA are in green and KHFCP (amino acids 104-108) are in yellow. Two more molecules of RhoA (brown) and Rho GDI (turquoise) are presented. The image is obtained from Protein Data Bank, PDB entry: 1CC0. (B) A ribbon representation of Cdc42 (yellow) and RhoGDI (lilac) complex. Side chain residues of Cdc42 that form interactions with Ser¹⁴⁸ and Tyr¹⁴⁴ of RhoGDI are shown (see text for explanation). This image has been obtained from Dr N. Keep (Birkbeck College, London).

Crystal structure of RhoA in complex with RhoGDI-1 (Fig. 6-11), shows that the closest portions of RhoA to be in contact with Ser¹⁴⁸ in RhoGDI are the YDRLRPL (switch II) and KHFCP regions. Multiple alignment analysis revealed that these sequences are conserved between Rho/Rac proteins (Fig. 6-15) and that they are loop sequences, again implying their important role. Sequence YDRLRPL contains several negatively charged residues and some bulky residues, which would not like the vicinity of a negatively charged phosphate. Both regions were found in Rac1 to form hydrogen bonds and hydrophobic interactions with Rho GDI (Grizot *et al.*, 2001). Namely, in the switch II region (residues 66-70) of Rac-1, the conserved amino acids Tyr⁶⁴, Arg⁶⁶, His¹⁰³ and His¹⁰⁴ form hydrogen bonds with GDI. Additionally, hydrophobic interactions involving Leu⁶⁷ and Leu⁷⁰ contribute to the stability of the Rac1-RhoGDI complex.

RHOA	MAAIRKKLVIVGDGACGKTCLLIVFSKDQFPEVYVPTVFENYVADIEVDGKQVELALWDT	60
RAC	MQAI--KCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVNLGLWDT	58
CDC42	MQTI--KCVVVGDGAVGKTCLLISYTTNKFPESEYVPTVFDNYAVTVMIGEPYTLGLFDT	58
	* : * * * : * * * * * * * * * : : * * * : * * * * : * : * : *	
RHOA	AGQED T RLR A SYPDTDVILMCFSIDSPDSLENIPEKWTPEVK F INVP IILVGNKKD	120
RAC	AGQED D RLRP S YPQTDVFLICFSLVSPASFENVRAKWYPEVR H ENTP IILVGTKLD	118
CDC42	AGQED D RLRP S YPQTDVFLVCFSVVSPSSFENVKEKWVPEIT H IKTPFLLVGTQID	118
	***** : * * : * : * : * : * : * : * : * : * : * : * : *	
RHOA	LRNDEHTRELAKMKQEPVKPEEGRDMANRIGAFGYMECSAKTKDGVREVFE MATRAALQ	180
RAC	LRDDKDTIEKLEKKLTPITYPQGLAMAKEIGAVKYLECSALTQRGKLTVFDEAIRAVLC	178
CDC42	LRDDPTIEKLAKNKQKPIPTETAELKARDLKAVKYVECSPTQKGLKNVFDEAILAEL	178
	** : * * : * : * * : * : * : * : * : * : * : * : * : *	
RHOA	ARRGKK-KSGCLIL 193	
RAC	PPPVKRRKRKCLLL 192	
CDC42	PPEPKK-SRRCVLL 191	
	** * - *	

Figure 6-15 *Multiple alignment of RhoGTPases.* Rat RhoA, Rac-1 and Cdc42 sequences were aligned using ClustalW. The regions suspected to be in contact with RhoGDI are marked in red with the conserved residues highlighted in black. The consensus sequence is labelled with asterisks and the conservative amino acid substitutions are identified by dots. Gi numbers are: RhoA-47605935, Rac-1-40354188 and Cdc42-24637541.

Phosphorylation of GDI on Ser¹⁴⁸ would for this reason destabilize the Rho-RhoGDI complex and enable dissociation.

The possible effect of the phosphorylation of Tyr¹⁴⁴ is less clear. This residue interacts with the C-terminal portion of the GTPase molecule (Fig. 6-14 B). This region contains basic side chains and is highly variable between small GTPase family members (Fig. 6-15).

6.4 Conclusions

- ET-1 stimulates the reorganization of actin in NRK-49F cells.
- ET-1 stimulates the phosphorylation of Ser¹⁴⁸ and Tyr¹⁴⁴ of RhoGDI-1 in NRK-49F cells.
- These residues appear to be important for the interaction of RhoGDI-1 with RhoGTPase proteins (based on their position in the protein (GDI-GTPase complex) and their evolutionary conservation).
- Additional phosphorylation of RhoGDI-1 both in resting and in ET-1 stimulated cells was observed. The exact position and significance of this remains to be discovered.
- An unidentified protein (Mw~80kDa), phosphorylated on Ser, co-precipitates with RhoGDI after ET-1 stimulation.

CHAPTER 7. SUMMARY AND FUTURE DIRECTIONS

7.1 Summary

The main findings presented in this thesis are as follows:

- ET-1 stimulates *de novo* protein synthesis in human lung fibroblasts;
- ET_AR and ET_BR are expressed by normal rat kidney fibroblasts and the binding of ET-1 to the ET_AR stimulates a rise in intracellular calcium levels;
- ET-1 stimulates the phosphorylation of c-Raf, ERK1/2 and p38MAPK;
- ET-1 modulates the phosphorylation of four different protein groups amongst which are the 5 new downstream phosphoprotein targets of ET-1 and a few previously reported phosphoproteins; and
- ET-1 stimulates the phosphorylation of Ser¹⁴⁸ and Tyr¹⁴⁴ of RhoGDI-1, which appears to influence the interactions of RhoGDI with small GTPases.

The main objective of this thesis was to generate a map of proteins modified by ET through monitoring two signalling effects of ET: gene expression and protein phosphorylation. The results obtained give an overview of the cellular processes modulated by ET and provide a framework for future studies.

In both proteomics studies (gene expression and protein phosphorylation) the same protein groups were affected by ET. These are: the cytoskeleton, chaperones, metabolic enzymes and regulatory proteins.

ET affects the cytoskeleton immediately by phosphorylation/dephosphorylation and by stimulating it to regroup (actin, chapter 6). These changes regulate mostly the

organization of the cytoskeleton (see chapter 5 discussion). Ultimately cytoskeletal rearrangements result in modulations of processes such as: cell adhesion, migration, contraction and vesicular transport. By inducing the synthesis of cytoskeletal proteins cells prepare probably for proliferation, in addition to the above mentioned processes.

The regulation of the level of chaperone proteins will have an impact on protein folding, assembly, secretion and cell survival pathways. ET alters the phosphorylation levels of this protein group and in this way probably affects protein-protein interactions and as yet unknown signalling mechanisms.

Cytoskeletal and chaperone proteins are the most abundant cell proteins whose members cover wide ranges of pI values and molecular weights. Therefore they are the most frequently encountered protein groups in the proteomics studies that use the global approach. However, this should not make these proteins less interesting, especially if their properties are changed as a result of an extracellular stimulus. On the other hand, the most attractive protein group are the regulatory proteins as they can tell us which intracellular pathways are activated and consequently which other proteins/molecules might be involved in the signal. Regulatory proteins are not abundant enough and in addition some of the most interesting ones (kinases) are concentrated in the basic end of the pI range. This makes it more difficult to identify them on a 2D gel.

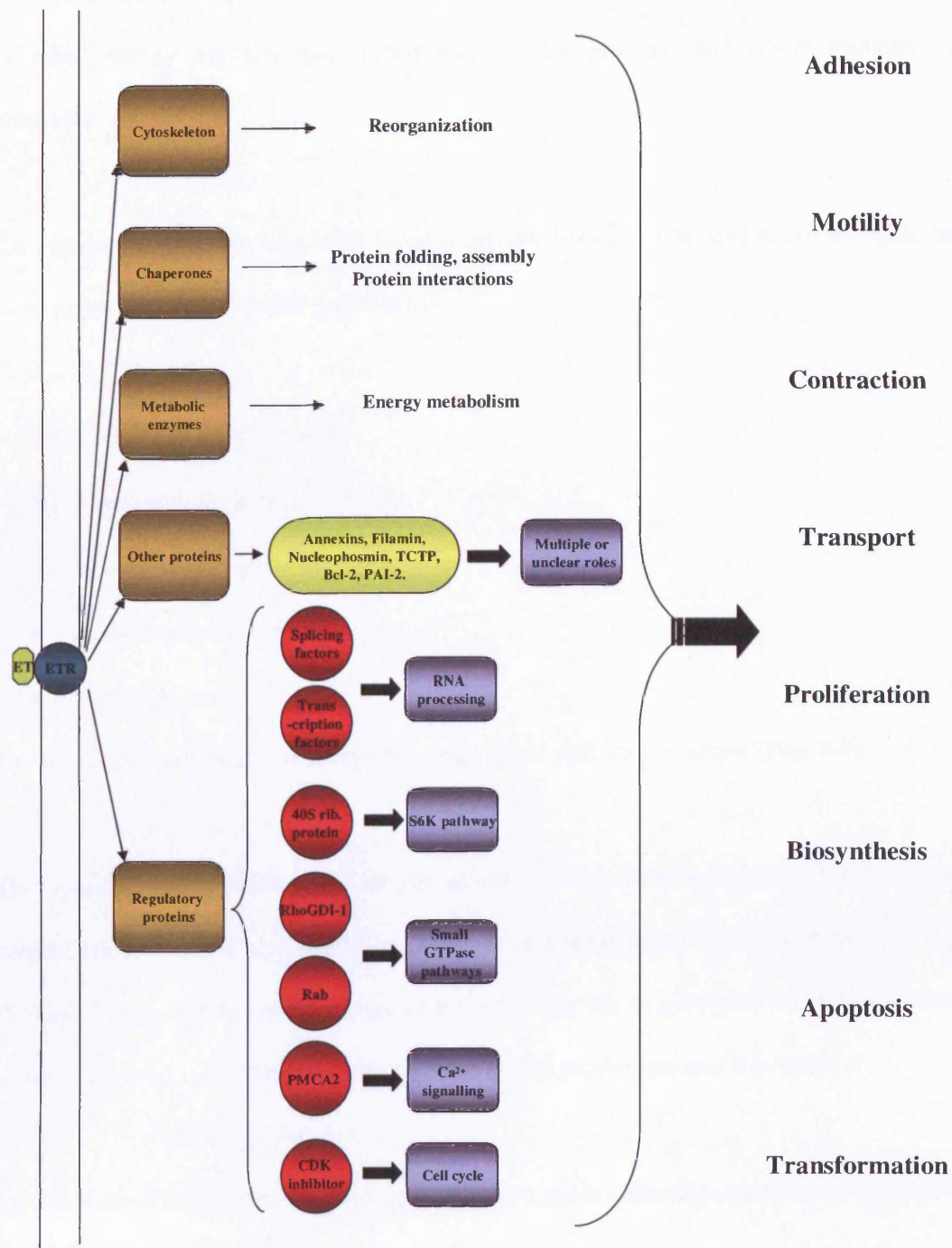


Figure 7-1 A schematic representation of the proteins and cellular processes regulated by ET.

By influencing the expression and phosphorylation of metabolic enzymes, ET controls the cells' energy supplies and accordingly the biosynthesis and active transport of molecules.

The regulatory proteins identified to be modulated by ET-1 in this thesis indicate the involvement of the following pathways:

- Ca^{2+} signalling;
- Small GTPase pathways;
- Ribosomal S6 kinase activity;
- RNA processing;
- Gene transcription; and
- MAPK signalling.

The implicated pathways converge to one or more cellular responses (Fig. 7-1).

The specific use of NRK cells for the studies of transforming growth factors was a helpful feature. ET-1 also has been shown to stimulate transformation of this cell type (Lahaye *et al.*, 1999). With this in mind, probably the majority of the ET stimulated events observed in this thesis prompt NRK cells to proliferate and transform.

Data obtained from proteomics experiments is a useful starting point for future studies. A more detailed investigation is needed to assign a specific function(s) for each protein in the network of ET signalling pathways.

7.2 Future studies

7.2.1 *RhoGDI*

ET-1 stimulated the phosphorylation of two sites in RhoGDI which were identified by nanospray sequencing. However, the results from this thesis suggested that RhoGDI is phosphorylated in a resting cell and that perhaps there are more phosphorylations/dephosphorylations happening after cell stimulation. A drawback of the experiments in this thesis was that the MALDI-TOF peptide analysis did not give a complete picture of the phosphorylation events (due to the poorer desorption and random dephosphorylation of phosphopeptides during the ionisation process).

A detailed peptide LC/MS/MS analysis of unstimulated and stimulated RhoGDI would be needed for the identification of all phosphorylation changes. The functional significance of these sites could then be verified by site directed mutagenesis. It would be very helpful if a specific antibody was raised against the identified phosphopeptides. By using other growth factors to stimulate the cells and using different cells it could be confirmed whether the pattern of the observed phosphorylations is a general rule or specific for the ET signal or the cell type. The final question is which of the small GTPases that the RhoGDI is known to bind to is affected by ET stimulation and GDI phosphorylation? Reverse co-immunoprecipitation experiments may be helpful in addressing this issue.

The observed reorganization of the actin cytoskeleton could be further verified and characterized by immunofluorescence microscopy. In addition specific small GTPase

inhibitors (like the C3-toxin selective for the RhoA protein) could help to establish the possible link between actin reorganization and small GTPase activation.

How can one go about identifying the 80kDa protein that co-precipitates with RhoGDI upon stimulation? This could be approached by separating the immunoprecipitated protein extract on a gradient 1D gel to achieve a better separation of the proteins. After immunodetection the protein could be cut directly from the nitrocellulose membrane digested with trypsin and the obtained peptides analysed by LC/MS/MS. The protein identity and its potential biological role could later be verified by immunodetection with the specific antibody if available, reverse co-immunoprecipitation, and additional experiments.

7.2.2 *MAPK signalling*

To help elucidate which ET receptors are responsible for the MAPK activation observed in this thesis specific receptor antagonists could be used. In addition an NRK-49F clone was generated in our laboratory where the expression of ET_AR was suppressed by the use of siRNA. This will enable the investigation of cellular responses generated by ET-1 binding to ET_BR, without the need to pharmacologically block the receptor.

The current information available about the signalling mechanisms that lead from GPCRs to p38MAPK is limited. Specific inhibitors of families of kinases and of protein phosphatases could be used to identify the enzymes relevant for the activation of p38MAPK. Subsequently, specific inhibitors of individual components of the relevant pathways could be used to delineate the signalling pathway to p38MAPK.

Proteins that co-precipitate with p38MAPK after stimulation could be identified by MALDI-TOF and LC-ESI mass spectrometry. In addition SILAC methodology could be exploited as follows: after labelling and cell stimulation a recombinant ATF-2 (a substrate for p38MAPK) could be used as bait to affinity purify activated p38MAPK and associated proteins. Proteins that interact with the bait in a stimulation-dependent manner would be more abundant and give more intense peaks in MALDI-TOF analysis. SILAC would enable the distinction from irrelevant peptides whose abundance would not change upon stimulation.

7.2.3 *Proteomics*

The proteins identified in this thesis are just a small proportion of the proteome modified by ET-1 stimulation. Limits of the detection were set by the pH range (pH 4-7), percentage of gel (11%) and buffers used for the solubilization of proteins. In addition to this, low abundant proteins could not be identified. In the future, the study started in this thesis could be broadened by using many different pH ranges in the first dimension of protein separation, different types of polyacrylamide gels (gradient gels, gels with larger separation distance) and different solubilization cocktails. Low abundant proteins would need to be enriched prior to identification. Multi-photon detection, which was recently used in our laboratory, should significantly aid the sensitive detection of low-abundance proteins (Kleiner *et al.*, 2005 in press).

In order to create statistics and overcome the problem of inter-gel variability several gels for one sample need to be run. More gels also means more sample load. This is a real issue when working with cultured cells, especially after IMAC phosphoprotein enrichment where the yield is 10-20% of the starting protein amount. In the future, it

would be helpful to work with larger quantities of starting material, such as large scale suspension based cell production.

In vivo studies or work with primary culture of cardiac myocytes and endothelial cells would be more useful for a better understanding of the physiological roles of ET and for clinical application.

The continuation of studies of protein isoforms would require methods that allow for the:

- accurate measurement of quantitative data; and
- MS characterization of complete protein molecules.

The former could be achieved easily with MPD. Complete protein analysis is at the moment achieved with FTICR, although this method is elaborate and far from straightforward. Proteomics still needs to improve methods for the resolution of complete proteins and to develop new MS methods for rapid isoform characterization.

APPENDIX

A. Proteins identified with MALDI-TOF peptide fingerprinting from

Table 3-1

Amino acid structure of identified proteins is shown with the sequences identified with MALDI-TOF highlighted in red.

s1: gi 4507877; Vinculin

```

1  MPVFHTRTIE SILEPVAQQI SHLVIMHEEG EVDGKAIPDL TAPVAAVQAA
51  VSNLVRVGKE TVQTTEQIL KRDMPPAFIK VENACTKLQV AAQMLQSDPY
101 SVPARDYLID GSRGILSGTS DLLLTFDEAE VRKIIRVCKG ILEYLTVAEV
151 VETMEDLVTY TKNLGPMTK MAKMIDERQQ ELTHQEHRVM LVNSMNTVKE
201 LLPVLISAMK IFVTTKNSKN QGIEEALKNR NFTLEKMSAE INEIIRVLQL
251 TSWDEDAWAS KDTEAMKRAL ASIDSKLNQA KGWLRDPSAS PGDAGEQAIR
301 QILDEAGKVG ELCAGKERRE ILGTCKMLGQ MTDQVADLRA RGQGSSPVAM
351 QKAQQVSQGL DVLTAQVENA ARKLEAMTNS KQSIKKIDA AQNWLADPNG
401 GPEGEEQIRG ALAEARKIAE LCDDPKERDD ILRSLGEISA LTSKLADLRR
451 QGKGDSPEAR ALAKQVATAL QNLQTKTNRA VANSRPAKAA VHLEGGIEQA
501 QRWIDNPTVD DRGVGQAAIR GLVAEGHRLA NVMMGPYRQD LLAKCDRVDQ
551 LTAQLADLAA RGEGESPOAR ALASQLQDSL KDLKARMQEA MTQEVSDVFS
601 DTTPIKLLA VAATAPPDAP NREEVFDERA ANFENHSGKL GATAEKAAAV
651 GTANKSTVEG IQASVKTARE LTPQVVSAAAR ILLRNPNQNA AYEHFETMKN
701 QWIDNVEKMT GLVDEAIDTK SLLDASEEAI KKDLCKCKVA MANIQPQMLV
751 AGATSIARRA NRILLVAKRE VENSEDPKFR EAVKAASDEL SKTISPMVMD
801 AKAVAGNISD PGLQKSFLDS GYRILGAVAK VREAFQPQEP DFPFPPPDLE
851 QLRLTDELAP PKPPLPEGEV PPPRPPPPPEE KDEEFPEQKA GEVINQPMMM
901 AARQLHDEAR KWSSKGNDII AAKRMALLM AEMSRLVRGG SGTKRALIQ
951 AKDIAKASDE VTRLAKEVAK QCTDKRIRTN LLQVCERIPT ISTQLKILST
1001 VKATMLGRIN ISDEESEQAT EMLVHNAQNL MQSVKETVRE AEAASIKIRT
1051 DAGFTLRWVR KTPWYQ

```

s15: gi 42476296; Tropomyosin 2

```

1  MDAIKKKMQM LKLDKENAID RAEQAEADKK QAEDRCKQLE EEQQALQKKL
51  KGTEDEVEKY SESVKEAQEK LEQAEKKATD AEADVASLNR RIQLVEEELD
101 RAQERLATAL QKLEEAEEKAA DESERGMKVI ENRAMKDEEK MELQEMQLKE
151 AKHIAEDSDR KYEEVARKLV ILEGELERSE ERAEVAESKC GDLEELKIV
201 TNNLKSLEAQ ADKYSTKEDK YEEEIKLEE KLKEAETRAE FAERSVAKLE
251 KTIDDLEDEV YAQMKYKAI SEELDNALND ITSL

```

s17: gi 136085; Tropomyosin 3

```

1  MEAIKKKMQM LKLDKENALD RAEQAEAEQK QAEERSKQLE DELAAMQKKL
51  KGTEDELDKY SEALKDAQEK LELAEEKKAAD AEAEVASLNR RIQLVEEELD
101 RAQERLATAL QKLEEAEEKAA DESERGMKVI ENRALKDEEK MELQEIQLKE
151 AKHIAEEADR KYEEVARKLV IIEGDLERTE ERAELAESKC SELEEEELKNV
201 TNNLKSLEAQ AEKYSQKEDK YEEEIKILTD KLKEAETRAE FAERSVAKLE
251 KTIDDLEDEL YAQKLKYKAI SEELDHALND MTSI

```

s29: gi 17986264; Myosin light polypeptide 6

```

1  MCDFTEDQTA DLIPSTEFKE AFQLFDRTGD GKILYSQCGD VMRALGQNPT
51 NAEVLKVLGN PKSDEMNVKV LDFEHFLPML QTVAKNKDQG TYEDYVEGLR
101 VFDKEGNGTV MGAEIRHVLV TLGEKMTEEE VEMLVAGHED SNGCINYEAF
151 VRHILSG

```

s5: gi 6470150; GRP78

```

1  MEEDKKEDVG TVVGIDLGTT YSCVGVFKNG RVEIIANDQG NRITPSYVAF
51 TPEGERLIGD AAKNQLTSNP ENTVFDKRL IGRTWNDPSV QQDIKFLPFK
101 VVEKKTkPYI QVDIGGGQTK TFAPEEISAM VLTKMKETAE AYLGGKVTHA
151 VVTVPAYFND AQRQATKDAG TIAGLNVMRI INEPTAAIA YGLDKREGEK
201 NILVFDLGGG TFDVSLTID NGVFEVVATN GDTHLGGEDEF DQRVMEHFIK
251 LYKKKTGKDV RKDNRAVQKL RREVEKAKRA LSSQHQARIE IESFYEGEDF
301 SETLTRAKEELNMDLFRST MKPVQKVLED SDLKKSDIDE IVLVGGSTRI
351 PKIQQLVKEF FNGKEPSRGI NPDEAVAYGA AVQAGVLSGD QDTGDLVLLD
401 VCPLTLGIET VGGVMTKLIP RNTVVPTKKS QIFSTASDNQ PTVTIKVYEG
451 ERPLTKDNHL LGTFDLTGIP PAPRGVPQIE VTFEIDVNGI LRVTAEDKGT
501 GNKNKITITN DQNRLTPEEI ERMVNDAEF AEEDKKLKER IDTRNELESY
551 AYSLKNQIGD KEKLGKGLSS EDKETMEKAV EEKIEWLESH QDADIEDFKA
601 KKKELEEIVQ PIISKLYGSA GPPPTGEEDT AELHHHHHH

```


s3: gi 2274968; Glucosidase

```

1  MAAVAAVAAR RRRSWASLVL AFLGVCLGIT LAVDRSNFKT CEESFCKRQ
51 RSIRPGLSPY RALLDSLQLG PDSLTVHLIH EVTKVLLVLE LQGLQKNMTR
101 FRIDELEPRR PRYRVPDVLV ADPPIARLSV SGRDENSVEL TMAEGPYKII
151 LTARPFRLDL LEDRSLLLSV NARGLLEFEH QRAPRVSQGS KDPAEGDGAQ
201 PEETPRDGDK PEETQGKAEK DEPGAWEETF KTHSDSKPYG PMSVGLDFSL
251 PGMEHVYGIF EHADNLRKLV TEGGEFYRLY NLDVFQYELY NPMALYGSVP
301 VLLAHNPHRD LGIFWLNAAE TWVDISSNTA GKTLFGKMMD YLQGSGETPQ
351 TDVRWMSETG IIDVFLLLGP SISDVFRQYA SLTGTQALPP LFSLGYHQSR
401 WNYRDEADVL EVDQGFDDHN LPCDVIWLDI EHADGKRYFT WDPSRFPQPR
451 TMLERLASKR RKLVAIVDPH IKVDSGYRVH EELRNLGLYV KTRDGS DYEG
501 WCWPGSAGYP DFTNPTMRAW WANMFSYDNY EGSAPNLFVW NDMNEPSVFN
551 GPEVTMLKDA QHYGGWEHRD VHNIYGLYVH MATADGLRQR SGGMERPFVL
601 ARAFFAGSQR FGAVWTGDNT AEWDHLKISI PMCLSLGLVG LSFCGADVGG
651 FFKNPEPELL VRWYQMGAYQ PFFRAHAHL D TGRREEPWLLP SQHNDIIRDA
701 LGQRYSLLEF WYTLLEYQHR EGIPVMRPLW VQYPQDVTF NIDDQYLLGD
751 ALLVHPVSDS GAHGVQVYLP GQGEVWYDIQ SYQKHGHPQT LYLPVTLSSI
801 PVFQRGGTIV PRWMRVRRSS ECMKDDPITL FVALSPQGT QGELFLDDGY
851 TFNYQTRQEF LLRRFSFSGN TLVSSADPE GHFETPIWIE RVVIIGAGKP
901 AAVVLQTKGS PESRSLFQHD PETSVLVLRK PGINVASDWS IHLR

```

s22: gi 189438; Cytochrome P-450-scc

```

1  HFYWELRQKG SVHHDYRGML YRLLGDSKMS FEDIKANVTE MLAGGVDTTS
51 MTLQWHLYEM ARNLKVQDML RAEVLAARHQ AQGDMATMLQ LVPLLKASIK
101 ETLRLHPISV TLQRYLVNDL VLRDYMIPAK TLVQVAIYAL GREPTFFDP
151 ENFDPTRWLS KDKNITYFRN LGFGWGVRC LGRRIA ELEM TIFLINMLEN
201 FRVEIQHLS D VGTTFNLI LLM PEKPISTFW PFNQEATQQ

```

s24: gi 852428; Ornithine decarboxylase antizyme

```

1  NPPCSGSSIA TASPERRKGI NPAPPSTPAA PCRSLACTAA AAAAVRVPGS
51 PSTAVVTRVR GLGGAPDAPH PPLKIPGGRG NSQRDHNLSA NLFYSDDRNL
101 VTEELTSNDK TRILNVQSRL TDAKRINWRT VLSGGSLYIE IPGGALPEGS
151 KDSFAVLLEF AEEQLRADHV FICFHKRED RAALLRTFSF LGFEIVRPGH
201 PLVPKRPDAC FMAYTFERES SGEEEE

```

s2: gi 55749531; Splicing factor 3B subunit 2

```

1 MATEHPEPPK AELQLPPPPP PGHYGAWAAQ ELQAKLAEIG APIQGNREEL
51 VERLQSYTRQ TGIVLNRPV LRGEDGDKAAP PFMSAQLPGI PMPPFPLGLP
101 PLQPPPPPPP PPPGLGLGFP MAHPPNLGPP PPLRVGEPVA LSEEERLKL
151 QQQAALLMQQ EERAKQQGDH SLKEHELLEQ QKRAAAVLLEQ ERQQEIAKMG
201 TPVPRPPQDM GQIGVRTPLG PRVAAPVGPV GPTPTVLPMG APVPRPRGPP
251 PPPGDENREM DDPSVGPKIP QALEKILQLK ESRQEEMNSQ QEEEEEMETDA
301 RSSLGQSASE TEEDTVSVSK KEKNRKRNR KKKKKPQVRV GVSSSSSGDR
351 EKDSTRSRGS DSPAADVEIE YVTEEPEIYE PNFIFFKRIF EAFKLTDDVK
401 KEKEKEPEKL DKLENSAAPK KKGFEHHKD SDDSSSDEQ EKKPEAPKLS
451 KKKLRMRNF TVAELKQLVA RPDVVMHDV TAQDPKLLVH LKATRNSVPV
501 PRHWCFFRKY LQKRGIEKP PFELPDFIKR TGIQEMREAL QEKEEQKTMK
551 SKMREKVRPK MGKIDIDYQK LHDAFFKWQT KPKLTIHGDL YYEGKEFETR
601 LKEKKPGDLS DELRISLGMP VGPNAHKVPP PWLIAMQRYG PPPSYPNLKI
651 PGLNSPIPES CSFGYHAGGW GKPPVDETCK PLYGDVFGTN AAEFQTKTEE
701 EEIDRTPWGE LEPSDEESSE EEEEESEDED KPDETGFITP ADSGLITPGG
751 FSSVPAGMET PELIELRKKK IEEAMDGSET PQLFTVLPEK RTATVGGAMM
801 GSTHIYDMST VMSRKGPAP LQGVEVALAP EELELDPAM TQKYEEHVRE
851 QQAQVEKEDF SDMVAEHAAK QKQKKRKAQP QDSRGGSKKY KEFKF

```

s4: gi 136652; Nucleolar transcription factor 1 (UBF-1)

```

1 MNGEADCPTD LEMAAPKGQD RWSQEDMLTL LECMKNNLPS NDSSKFKTTE
51 SHMDWEKVAF KDFSMDMCKL KWVEISNEVR KFRTLTELIL DAQEHVKNPY
101 KGKKLKKHPD FPKKPLTPYF RFFMEKRAKY AKLHPEMSNL DLTKILSKKY
151 KELPEKKMK YIQDFQREKQ EFERNLARFR EDHPDLIQNA KKSDIEPKP
201 TPQQLWYTHE KKVYLKVRPD EIMRDYIQKH PELNISEEGI TKSTLTKAER
251 QLKDKFDGRP TKPPPNYSYL YCAELMANMK DVPSTERMVL CSQWKLLSQ
301 KEKDAYHKKC DQKKKDYEVE LLRFLESLPE EEQQRVLGEE KMLNINKKQA
351 TSPASKKPAQ EGGKGGSEKP KRPVSAMFIF SEEKRRQLQE ERPELSESEL
401 TRLLARMWND LSEKKKAKYK AREAALKAQS ERKPGGEREE RGKLPESPKR
451 AEEIWQQSVI GDYLARFKND RVKALKAMEM TNNMEKKEK LMWIKKAAED
501 QKRYERELSE MRAPPAATNS SKMKFQGEP KKPPMNGYQK FSQELLSNGE
551 LNHLPLKERM VEIGSRWQRI SQQKEHYKK LAEEQQQYK VHLDLWVKSL
601 SPQDRAAYKE YISNKRKSM TLRGPNPKSS RTTLQSKSES EEDDEEDED
651 EDEDEEEEDD ENGDSSEDDG DSSESSSEDE SEDGDENEED DEDEDDEDED
701 DEDEDNESEG SSSSSSSSGD SSDSDSN

```

s14:gi 1881852 ;SRY (sex determining region Y)-box 5

```

1 MPALRINSGA GPLKASVPAA LASPSARVST IGYLNDHDAV TKAIQEARQM
51 KEQLRREQV LDGKVAVVNS LGLNNCRTEK EKTTLESLTQ QLAVKQQEG
101 KFSHAMMDFN LSGSDGSAG VSESRIYRES RGRGSNEPHI KRPMNAFMVW
151 AKDERRKILQ AFPDMHNSNI SKILGSRWKA MTNLEKQPYY EEQARLSQH
201 LEKYPDYKY PRPKRTCLVD GKKLRIGEYK AIMRNRRQEM RQYFNVQQA
251 QIPIATAGVV YPGAIAMAGM PPHLPSEHS SVSSSPEPGM PVIQSTYGVK
301 GEEPHIKEEI QAEDINGEY DEYDEEEDDP DVDYGSDSEN HIAGQAN

```


s23: gi 20379074; Ras-related protein Rab-14 (long)

```

1  MATTPYNYSY IFKYIIIGDM GVGKSCLLHQ FTEKKFMADC PHTIGVEFGT
51  RIIEVSGQKI KLQIWDTAGQ ERFRAVTRSY YRGAAGALMV YDITRRSTYN
101 HLSSWLT DAR NLTNPNTVII LIGNKADLEA QRDVTYEEAK QFAEENGLLF
151 LEASAKTGEN VEDAFLEAAK KIYQNIQDGS LDLNAAESGV QHKPSAPQGG
201 RLTSEPQPQR EGC GC

```

s28: gi 6563200; Ras-related protein Rab-14 (short)

```

1  MAT PYNYSY IFKYIIIGDM GVGKSCLLHQ FTEKKFMADC PHTIGVEFGT
51  RIIEVSGQKI KLQIWDTAGQ ERFRAVTRSY YRGAAGALMV YDITRRSTYN
101 HLSSWLT DAR NLTNPNTVII LIGNKADLEA QRDVTYEEAK QFAEENGLLF
151 LEASAKTGEN VEDAFLEAAK KIYQNIQDGS LDLNAAESGV QHKPSAPQGG
201 RLTSEPQPQR EGC GC

```

s31: gi 4506367 RAB3A, member RAS oncogene family

```

1  MASATDSRYG QKESDQNF D YMFKILIIGN SSVGKTSFLF RYADDSFTPA
51  FVSTVGIDFK VKTIYRNDKK IKLQIWDTAG QERYRTITTA YRGAAGALMV
101 MYDITNEESF NAVQDWSQI KTYSWDNAQV LLVGNKCDME DERVVSSERG
151 RQLADHLGFE FFEASAKDNI NVKQTFERLE DVICEKMSSES LDTADPAVTG
201 AKQGPQLSDQ QVPPHQDCAC

```

s33: gi 125969; 40S ribosomal protein SA; laminin-binding protein

```

1  SGALDVLQMK EEDVLKFLAA GTHLGSTNLD FQMEQYIYKR KSDGIYIINL
51  KRTWEKLLLA ARAIVAIENP ADVSVISSRN TGQRAVLKFA AATGATPIAG
101 RFTPGTFTNQ IQAAFREPR L VVTDPRADH QPLTEASYVN LPTIALCNTD
151 SPLRYVDIAI PCNNKGAHSV GLMWWMLARE VLRMRGTISR EHPWEVMPDL
201 YFYRDPEEIE KEEQAAAEKA VTKEEFQGEW TAPAPEFTAT QPEVADWSEG
251 VQVPSVPIQQ FPTEDWSAQP ATEDWSAAPT AQATEWVGAT TDWS

```

s35: gi 1168873; Cyclin-dependent kinase inhibitor 1B

```

1  MSNVRVSN GS PSLERMDARQ AEHPKPSACR NLFGPVDHEE LTRDLEKHC R
51  DMEEASQRKW NFDFQNHKPL EGKYEWEVE KGSLPEFYR PPRPPKGACK
101 VPAQESQDVS GSRPAAPLIG APANSEDTL VDPKTDP SDS QTGLAEQCAG
151 IRKRPATDDS STQNK RANRT EENVSDGSPN AGSVEQTPKK PGLRRRQT

```

s7: gi 938227; Filamin

```

1  VVGKSADFVV EAIGTEVGT L GFSIEGPSQA KIECDDKGDG SCDVRYWPTE
51  PGEYAVHVIC DDEDIRDS PF IAHILPAPPD CFPDKVKA FG PGLEPTGCIV
101 DKPAEFTIDA RAAGKGDLKL YAQDADGCPI DIKVIPNGNG TFRCSYVPTK
151 PIKHTIIISW GGVNVKSPF RVNVGEGSHP ERVKVYGP GV EKTASRPMSP
201 PTFTVDCSEA GQGDV SIGIK CAPGVVGPAE A

```

s11: gi 1352712 Plasminogen activator inhibitor-2 precursor

```

1 MEDLCVANTL FALNLFKHLA KASPTQNLFL SPWSISSTMA MVYMGSRGST
51 EDQMAKVLQF NEVGANAVTP MTPENFTSCG FMQQIQKGSY PDAILQAQAA
101 DKIHSSFRSL SSAINASTGN YLLESVNKLF GEKSASFEE YIRLCQKYYS
151 SEPQAVDFLE CAEEARKKIN SWVKTQTKGK IPNLLPEGSV DGDTRMVLVN
201 AVYFKGKWK T PFEKKLNGLY PFRVNSAQR PVQMMYLREK LNIGYIEDLK
251 AQILELPYAG DVSMFLLLPD EIADVSTGLE LLESEITYDK LNKWTSKDKM
301 AEDEVYIP QFKLEEHYEL RSILRSMGME DAFNKGRANF SGMSEKNDLF
351 LSEVFHQAMV DVNEEGTEAA AGTGGVMTGR TGHGGPQFVA DHPFLFLIMH
401 KITNCILFFG RFSSP

```

s20: gi 1703319; Annexin 4

```

1 MATKGGTVKA ASGFNAMEDA QTLRKAMKGL GTDEDAIISV LAYRNTAQRQ
51 EIRTAYKSTI GRDLIDDLKS ELSGNFEQVI VGMPTPTVLY DVQELRRAMK
101 GAGTDEGCLI EILASRTPEE IRRISQTYQQ QYGRSLEDDI RSDTSFMFQR
151 VLVSLSAGGR DEGNLYDDAL VRQDAQDLYE AGEKKWGTDE VKFLTIVLCSR
201 NRNHLLHVFD EYKRISQKDI EQSIKSETSG SFEDALLAIV KCMRNKSAYF
251 AEKLYKSMKG LGTDDNTLIR VMVSRAEIDM LDIRAHFKRL YGKSLYSFIK
301 GDTSGDYRKV LLVLCGGDD

```

s25: gi 136479; Tumour protein, translationally-controlled 1

```

1 MIIYRDLISH DEMFSDIYKI REIADGLCLE VEGKMVSRT GNIDDSLIGG
51 NASAEGPEGE GTESTVITGV DIVMNHHLQE TSFTKEAYKK YIKDYMKSII
101 GKLEEQRPER VKPFMTGAAE QIKHILANFK NYQFFIGENM NPDGMVALLD
151 YREDGVTPYM IFFKDGLEME KC

```

s26: gi 4757840; Bcl2-related protein A1

```

1 MTDCEFGYIY RLAQDYLCV LQIPQPGSGP SKTSRVLQNV AFSVQKEVEK
51 NLKSCLDNVN VVSVDTARTL FNQVMEKEFE DGIINWGRIV TIFAFEGILI
101 KLLRQQIAP DVDTYKEISY FVAEFIMNNT GEWIRQNGGW ENGFVKKFEP
151 KSGWMTFLEV TGKICEMLSL LKQYC

```

s30: gi 825671; Nucleophosmin

```

1 QNYLFGCELK ADKDYHFKVD NDENEHQLSL RTVSLGAGAK DELHIVEAEA
51 MNYEGSPIKV TLATLKMSVQ PTVSLGGFEI TPPVVLRLKC GSGPVHISGQ
101 HLVAVEEDAE SEDEEEEDVK LLSISGKRSA PGGGSKVPQK KVKLAADDD
151 DDDDEEDDDE DDDDDDFDDE EAEEKAPVKK SIRDTPAKNA QKSNQNGKDS
201 KPSSTPRSKG QESFKKQEK PKTPKGPSSV EDIKAKMQAS IEKGGSLPKV
251 EAKFINYVKN CFRMTDQEI QDLWQWRKSL

```


B. Proteins identified with MALDI-TOF peptide fingerprinting from Table 5-1

Sequences of proteins from table 5-1 are shown. All sequences are obtained from rat unless otherwise specified. The peptides identified by mass spectrometry and database searches are displayed in red. If a protein was identified from an organism other than rat, closest sequences from rat are also shown. The differences (between the sequence from rat and the sequence from other species) if present, are highlighted in black.

s11 (also s10): gi 809561; gamma-actin [Mus musculus]

```

1 LVIDNGSGMC KAGFAGDDAP RAVFPSIVGR PRHQGVMMVGM GQKDSYVGDE
51 AQSKRGILTL KYPIEHGIVT NWDDMEKIWH HTFYNELRVA PEEHPVLLTE
101 APLNPKANRE KMTQIMFETF NTPAMYVAIQ AVLSLYASGR TTGIVMDSGD
151 GVTHTVPIYE GYALPHAILR LDLAGRDLDL YLMKILTERG YSFTTTAERE
201 IVRDIKEKLC YVALDFEQEM ATAASSSSLE KSYELPDGQV ITIGNERFRC
251 PEALFQPSFL GMESCGIHET TFNSIMKCDV DIRKDLANT VLSGGTTMYP
301 GIADRMQKEI TALAPSTMKI KIIAPPERKY SVWIGGSILA SLSTFQQMWI
351 SKQEYDESGP SIVHRKCF

```

gi 34856512; similar to gamma actin-like protein [Rattus norvegicus]

```

1 MEEEIAALVI DNGSGMCKAG FAGDDAPRAV FPSIVGRPRH QGVMVGMGQK
51 DSYVGDEAQS KRGILTLKYP IEHGIVTNWD DMEKIWHHTF YNELRVAPEE
101 HPVLLTEAPL NPKANREKMT QIMFETFNTF AMYVAIQAVL SLYASGRTTG
151 IVMDSGDGVT HTVPIYEGYA LPHAILRLDL AGRDLTDYLM KILTERGYSF
201 TTTAEREIVR DIKEKLCYVA LDFAQEMATA ASSSSLEKSY ELPDQGVITI
251 GNERFRCPEA LFQPSFLGME SCGIHETTFN SIMKCDVDIR KDLYANTVLS
301 GGTTMYPGIA DRMQKEITAL APSTMKIKII APPERKYSVW IGGSILASLS
351 TFQQMWISKQ EYDEQAPPLS TANASRWTEQ VPGICCMS

```

gi 13592133; cytoplasmic beta-actin [Rattus norvegicus]

```

1 MDDIAALVV DNGSGMCKAG FAGDDAPRAV FPSIVGRPRH QGVMVGMGQK
51 DSYVGDEAQS KRGILTLKYP IEHGIVTNWD DMEKIWHHTF YNELRVAPEE
101 HPVLLTEAPL NPKANREKMT QIMFETFNTF AMYVAIQAVL SLYASGRTTG
151 IVMDSGDGVT HTVPIYEGYA LPHAILRLDL AGRDLTDYLM KILTERGYSF
201 TTTAEREIVR DIKEKLCYVA LDFAQEMATA ASSSSLEKSY ELPDQGVITI
251 GNERFRCPEA LFQPSFLGME SCGIHETTFN SIMKCDVDIR KDLYANTVLS
301 GGTTMYPGIA DRMQKEITAL APSTMKIKII APPERKYSVW IGGSILASLS
351 TFQQMWISKQ EYDESGPSIV HRKCF

```

s4:gi 13242237; heat shock protein 8; Heat shock cognate protein 70; heat shock 70kD protein 8 [Rattus norvegicus]

```

1  MSKGPVAGID LGTTYSCVGV FQHGKVEIIA NDQGNRTTPS YVAFTDTERL
51 IGDAAKNQVA MNPTNTVFDA KRLIGRRFDD AVVQSDMKHW PFMVVNDAGR
101 PKVQVEYKGE TKSFYPEEVS SMVLTKMKEI AEAYLGKTVT NAVVTVPAYF
151 NDSQRQATKD AGTIAGLNVL RIINEPTAAA IAYGLDKKVG AERNVLIFDL
201 GGGTFDVSIL TIEDGIFEVK STAGDTHLGG EDFDNRMVNH FIAEFKRKHK
251 KDISENKRAV RRLRTACERA KRTLSSSTQA SIEIDSLYEG IDFYTSITRA
301 RFEELNADLF RGTLDPVEKA LRDAKLDSQ IHDIVLVGGS TRIPKIQKLL
351 QDFFNGKELN KSINPDEAVA YGAAVQAAIL SGDKSENVQD LLLLDVTPLS
401 LGIETAGGVM TVLIKRNTTI PTKQTQTFTT YSDNQPGVLI QVYEGERAMT
451 KDNLLGKFE LTGIPPAPRG VPQIEVTFDI DANGILNVSA VDKSTGKENK
501 ITITNDKGRL SKEDIERMVQ EAEKYKAEDE KQDKVSSKN SLESYAFNMK
551 ATVEDEKLQG KINDEDKQKI LDKCNEIISW LDKNQTAKEE EFEHQQKELE
601 KVCNPIITKL YQSAGGMPGG MPGGFPGGGA PPSGGASSGP TIEEVD

```

gi 123647; Heat shock cognate 71 kDa protein [Cricetulus griseus]

```

1  MSKGPVAGID LGTTYSCVGV FQHGKVEIIA NDQGNRTTPS YVAFTDTERL
51 IGDAAKNQVA MNPTNTVFDA KRLIGRRFDD AVVQSDMKHW PFMVVNDAGR
101 PKVQVEYKGE AKSFYPEEVS SMVLTKMKEI AEAYLGKTVT NAVVTVPAYF
151 NDSQRQATKD AGTIAGLNVL RIINEPTAAA IAYGLDKKVG AERNVLIFDL
201 GGGTFDVSIL TIEDGIFEVK STAGDTHLGG EDFDNRMVNH FIAEFKRNDK
251 KDISENKRAV RRLRTACERA KRTLSSSTQA SIEIDSLYEG IDFYTSITRA
301 RFEELNADLF RGTLDPVEKA LRDAKLDSQ IHDIVLVGGS TRIPKIQKLL
351 QDFFNGKELN KSINPDEAVA YGAAVQAAIL SGDKSENVQD LLLLDVTPLS
401 LGIETAGGVM TVLIKRNTTI PTKQTQTFTT YSDNQPGVLI QVYEGERAMT
451 KDNLLGKFE LTGIPPAPRG VPQIEVTFDI DANGILNVSA VDKSTGKENK
501 ITITNDKGRL SKEDIERMVQ EAEKYKAEDE KQDKVSSKN SLESYAFNMK
551 ATVEDEKLQG KINDEDKQKI LDKCNEIISW LDKNQTAKEE EFEHQQKELE
601 KVCNPIITKL YQSAGGMPGG MPGGFPGGGA PPSGGASSGP TIEEVD

```


s4a: gi 806907; FK506 binding protein 10; FK506 binding protein 6 (65 kDa); FK506 binding protein 10 (65 kDa) [Mus musculus]

```

1 MFLVGSSSHT LHRVRILPLL LLLQTLERGL GRASPAGAPL EDVVIERYHI
51 PRACPREVQM GDFVRYHYNG TFEDGKKFDS SYDRSTLVAI VVGVGRLITG
101 MDRGLMGMCV NERRRLLIVPP HLGYSIGVA GLIPPDATLY FDVVLDDVWN
151 KADTVQSTIL LRPPYCPRMV QNSDFVRYHY NGTLLDGTGF DNSYSRGGTY
201 DTYIGSGWLI KGMDQGLLGM CPGEKRKIII PPFLAYGEKG YGTVIPPQAS
251 LVFYVLLLDV HNPKDTVQLE TLELPQGCVR RAVAGDFMRY HYNGLMDGT
301 LFDSSYSRNH TYNTYVGQGY IIPGMDQGLQ GACIGERRRI TVPPHLAYGE
351 NGTGDKIPGS AVLIFDVHVI DFHNPSDPVE IKTLRPPEN CNETSKIGDF
401 IRYHYNCSSL DGTRLFSSHD YEAPQEITLG ANKVIEGLDR GLQGMCVGER
451 RQLIVPPHLA HGENGARGVP GSAVLLFEVE LVSREDGLPT GYLFVWYQDP
501 STSLFEDMDL NKDGEVPPEE FSSFIKAQVN EGKGRLMPGQ DPKTISDMF
551 QNQDRNQDGK ITAEELKLKS DEDQERVHEE L

```

gi 34873708; similar to 65kDa FK506-binding protein [Rattus norvegicus]

```

1 MFLVGAPSHT LPRLHILPLL LLLQTLERGL GRASPSGAPL EDVVIERYHI
51 PRACPREVQM GDFVRYHYNG TFEDGKKFDS SYDRSTLVAI IVGVGRLITG
101 MDRGLMGMCV NERRRLIIPP HLGYSIGLA GLIPPDATLY FDVVLDDVWN
151 KEDTVQSTIL LRPPYCPRMV RNSDFVRYHY NGTLLDGTAF DSSYSRGGTY
201 DTYIGSGWLI KGMDQGLLGM CPGEKRKIII PPFLAYGEKG YGTVIPPQAS
251 LVFYVLLIDV HNPKDTVQLE TLELPQDCVR RAVAGDFMRY HYNGLMDGT
301 LFDSRSGVHP EGYIIPGMDQ GLQGACIGER RRITVPPHLA YGENGTGDKI
351 PGS AVLIFDV HVIDFHNPAD PVEIKTLFRP PESCNETAKI GDFIRYHYNC
401 SLLDGTRLF S

```

s3: gi 87528; dnaK-type molecular chaperone HSPA5 precursor [Homo sapiens]

```

1  MKLSLVAAML LLLSAAAE AEE EDKKEDVGTV VGIDLGTTYS CVGVFKNGRV
51 EIIANDQGNR ITPSYVAFTP EGERLIGDAA KNQLTSNPEN TVFDAKRLIG
101 RTWNDPSVQQ DIKFLPFKVV EKKTKPYIQV DIGGGQTKTF APEEISAMVL
151 TKMKETAEEY LGKKVTHAVV TVPAYFNDAQ RQATKDAGTI AGLNVMRIIN
201 EPTAAAIAYG LDKREGEKNI LVFDLGGGTF DVSLLTIDNG VFEVVATNGD
251 THLGGEDFDQ RVMEHFIKLY KKKTGKDVRK DNRAVQKLRR EVEKAKALSS
301 QHQARIEIES FYEGEDFSET LTRAKFEELN MDLFRSTMKP VQKVLESDSL
351 KKSDIDEIVL VGGSTRIPKI QQLVKEFFNG KEPSRGINPD EAVAYGAAVQ
401 AGVLSGDQDT GDLVLLHVCP LTLGIETVGG VMTKLIPSNT VVPTKNSQIF
451 STASDNQPTV TIKVYEGERP LTKDNHLLGT FDLTGIPPAP RGVPQIEVTF
501 EIDVNGILRV TAEDKGTGNK NKITITNDQN RLTPEEIERM VNDAEKFAEE
551 DKKLKERIDT RNELESYAYS LKNQIGDKEK LGGKLSSEDK ETMEKAVEEK
601 IEWLESHQDA DIEDFKAKKK ELEEIVQPII SKLYGSAGPP PTGEEDTAEK
651 DEL

```

gi 25742763; heat shock 70kD protein 5 [Rattus norvegicus]

```

1  MKFTVVAAL LLLCAVRAEE EDKKEDVGTV VGIDLGTTYS CVGVFKNGRV
51 EIIANDQGNR ITPSYVAFTP EGERLIGDAA KNQLTSNPEN TVFDAKRLIG
101 RTWNDPSVQQ DIKFLPFKVV EKKTKPYIQV DIGGGQTKTF APEEISAMVL
151 TKMKETAEEY LGKKVTHAVV TVPAYFNDAQ RQATKDAGTI AGLNVMRIIN
201 EPTAAAIAYG LDKREGEKNI LVFDLGGGTF DVSLLTIDNG VFEVVATNGD
251 THLGGEDFDQ RVMEHFIKLY KKKTGKDVRK DNRAVQKLRR EVEKAKRALS
301 SQHQARIEIE SFFEGEDFSE TLTRAKFEEL NMDLFRSTMK PVQKVLESDS
351 LKKSDIDEIV LVGGSTRIPK IQQLVKEFFN GKEPSRGINP DEAVAYGAAV
401 QAGVLSGDQD TGDVLLDVC PLTLGIETVG GVMTKLIPRN TVVPTKKSQI
451 FSTASDNQPT VTIKVYEGER PLTKDNHLLG TFDLTGIPPA PRGVPQIEVT
501 FEIDVNGILR VTAEDKGTGN KNKITITNDQ NRLTPEEIER MVNDAEKFAE
551 EDKKLKERID TRNELESYAY SLKNQIGDKE KLGGKLSPED KETMEKAVEE
601 KIEWLESHQD ADIEDFKAKK KELEEIVQPI ISKLYGSGGP PPTGEEDTSE
651 KDEL

```

gi 31981722; heat shock 70kD protein 5 (glucose-regulated protein); glucose regulated protein, 78 kDa; heat shock 70kD protein 5 (glucose-regulated protein, 78kD) [Mus musculus]

```

1  MMKFTVVAAL LLLLGAVRAE EEDKKEDVGT VVGIDLGTTY SCVGVFKNGR
51 VEIIANDQGN RITPSYVAFT PEGERLIGDA AKNQLTSNPE NTVFDAKRLI
101 GRTWNDPSVQ QDIKFLPFKV VEKKTTKPYIQ VDIGGGQTKT FAPEEISAMV
151 LTKMKETAEE YLGKKVTHAV VTPPAYFNDA QRQATKDAGT IAGLNVMRII
201 NEPTAAAIAY GLDKREGEKN ILVFDLGGGT FDVSLLTIDN GVFEVVATNG
251 DTHLGGEDFD QRVMEHFIKL YKKKTGKDVR KDNRAVQKLRR REVEKAKRAL
301 SSQHQARIEI ESFFEGEDFS ETLTRAKFEE LNMDLFRSTM KPVQEVLEDS
351 DLKKSDIDEI VLVGGSTRIP KIQQLVKEFF NGKEPSRGIN PDEAVAYGAA
401 VQAGVLSGDQ DTGDLVLLDV CPLTLGIETV GGVMTKLIPR NTVVPTKKSQ
451 IFSTASDNQP TVTIKVYEGE RPLTKDNHLL GTFDLTGIPP APRGVPQIEV
501 TFEIDVNGII RVTAEDKGTG NKNKITITND QNRLTPEEIE RMVNDAEKFA
551 EEDKKLKERI DTRNELESYA YSLKNQIGDK EKLGGKLSSE DKETMEKAVE
601 EKIEWLESHQ DADIEDFKAK KKELEEIVQP IISKLYGSGG PPPTGEEDTS
651 EKDEL

```


s21: gi 33859596; procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide [Mus musculus]

```

1 MIWVVLMMAI LLPQSLAHPG FFTSIGQMTD LIHNEKDLVT SLKDYIKAE
51 DKLEQIKKWA EKLDRLTSTA TKDPEGFVGH PVNAFKLMKR LNTEWSELEN
101 LILKDMSDGF ISNLTIQRQY FPNDQDQVGA AKALFRLQDT YNLDTNTISK
151 GNLPGVQHK S FLTAEDCFEL GK VAYTEADY YHTELWMEQA LTQLEEGELS
201 TVDKVSVLDY LSYAVYQQGD LDKALLLTKK LLELDPEHQ ANGNLVYFEY
251 IMSKEKDANK SASGDQSDQK TAPKKKGIAV DYLPERQKYE MLCRGEGIKM
301 TPRRQKR LFC RYHDGNNRNP FILAPAKQED EWDKPRIIRF HDIISDAEIE
351 IVKDLAKPRL RRATISNPVT GALETVHYRI SKSAWLSGYE DPVVSRIINMR
401 IQDLTGLDVS TAEELQVANY GVGGQYEPHF DFARKDEPDA FRELGTGNRI
451 ATWLFYMSDV SAGGATVFPE VGASVWPKKG TAVFWYNLFA SGECDYSTRH
501 AACPVLVGNK WVSNNKWLHER GQEFRPCTL SELE

```

gi 25453386; prolyl 4-hydroxylase alpha subunit [Rattus norvegicus]

```

1 MIWGVLMGGI LLPQCSAHPG FFTSIGQMTD LIHNEKDLVT SLKDYIKAE
51 DKLEQIKKWA EKLDRLTSTA TKDPEGFVGH PVNAFKLMKR LNTEWSELEN
101 LILKDMSDGF ISNLTIQRQY FPNDQDQVGA AKALFRLQDT YNLDTNTISK
151 GNLPGVKKHS FLTAEDCFEL GK VAYTEADY YHTELWMEQA LMQLEEGEMS
201 TVDKVSVLDY LSYAVYQQGD LDKALLLTKK LLELDPEHQ ANGNLVYFEY
251 IMSKEKDANK SASGERADQK TTPKKKGIAV DYLPERQKYE MLCRGEGIKM
301 TPRRQKR LFC RYHDGNNRNP FILAPAKQED EWDKPRIIRF HDIISDAEIE
351 IVKDLAKPRL SRATVHDPET GKLTTAQYRV SKSAWLSGYE DPVVSRIINMR
401 IQDLTGLDVS TAEELQVANY GVGGQYEPHF DFARKDEPDA FRELGTGNRI
451 ATWLFYMSDV SAGGATVFPE VGASVWPKKG TAVFWYNLFA SGECDYSTRH
501 AACPVLVGNK WVSNNKWLHER GQEFRPCTL SELE

```

s1: gi 202549; Protein disulfide isomerase chain 1

```

1 VLVLKKSNEA EALAAHNYLL VEFYAPWCGH CKALAPEYAK AAKLKAE
51 EIRLAKVDAT EESDLAQYQ VRGYPTIKFF KNGDTASPKE YTAGREADDI
101 VNWLKKRTGP AATTLSDTAA AESLVDSSSE TVIGFFKDAG SDSAKQFLLA
151 AEAVDDIPFG ITSNSDVFSK YQLDKDGVVL FKKFDEGRNN FEGETKEKL
201 LDFIKHNQLP LVIEFTEQTA PKIFGGEIKT HILLFLPKSV SDYDGKLSNF
251 KKAAGFGK GK ILFIFIDS DH TDNQRIEFF GLKKEECPAV RLITLEEEMT
301 KYKPESEDEL AEKITQFCHH FLEGKIKPHL MSQELPEDWD KQPVKVLVGK
351 NFEEVAFDEK KNVFVEFYAP WCGHCKQLAP IWDKLGETYK DHENIVIAKM
401 DSTANEVEAV KVHSFPTLKF FPASADRTVI DYNGERTLDG FKKFLES
451 DGAGDNDLDD LEEALEPDME EDDDQKAVKD EL

```

s2: gi 25742763; 78 kDa glucose-regulated protein precursor (GRP78)

```

1 MKFTVVAAL LLLCAVRAEE EDKKEDVGT V GIDLGTTYS CVGVFKNGRV
51 EIIANDQGNR ITPSYVAFTP EGERLIGDAA KNQLTSNPEN TVFDAKRLIG
101 RTWNDPVSQQ DIKFLPFKV EKKTKPYIQV DIGGGQTKTF APEEISAMVL
151 TKMKETAEEY LGKKVTHAVV TVPAYFNDAQ RQATKDAGTI AGLNVMRIIN
201 EPTAAAIAYG LDKREGEKNI LVFDLGGGTF DVSLLTIDNG VFEVVATNGD
251 THLGGEFDQ RVMEHFILY KKKTGKDVRK DNRAVQKLRR EVEKAKRALS
301 SQHQARIEIE SFFEGEDFSE TLTRAKFEEL NMDLFRSTMK PVQKVLSDSD
351 LKKSIDIIEV LVGGSTRIPK IQQLVKEFFN GKEPSRGINP DEAVAYGAAV
401 QAGVLSGDQD TGDVLVLDVC PLTLGIETVG GVMTKLIPRN TVVPTKKSQI
451 FSTASDNQPT VTIKVYEGE PLTKDNHLLG TFDLTGIPPA PRGVPQIEVT
501 FEIDVNGIIR VTAEDKGTGN KNKITITNDQ NRLTPEEIER MVNDAEKFAE
551 EDKKLKERID TRNELESYAY SLKNQIGDKE KLGKLSPEDE KETMEKAVEE
601 KIEWLESHQD ADIEDFKAKK KELEEIVQPI ISKLYGSGGP PPTGEEDTSE
651 KDEL

```

s5: gi 13242237; Heat shock cognate 71kDa protein

```

1 MSKGPVAGID LGTTYSCVGV FQHGKVEIIA NDQGNRTTPS YVAFDTERL
51 IGDAAKNQVA MNPTNTVFDA KRLIGRRFDD AVVQSDMKHW PFMVNDAGR
101 PKVQVEYKGE TKSFYPEEVS SMVLTKMKEI AEAYLGKTVT NAVVTVPAYF
151 NDSQRQATKD AGTIAGNLVL RIINEPTAAA IAYGLDKKVG AERNVLIFDL
201 GGGTFDVSIL TIEDGIFEVK STAGDTHLGG EDFDNRMVNH FIAEFKRKHK
251 KDISENKRAV RRLRTACERA KRTLSSSTQA SIEIDSLYEG IDFYTSITRA
301 RFEELNADLF RGTLDPEKA LRDAKLDKSQ IHDIVLVGG TRIPKIQKLL
351 QDFFNGKELN KSINPDEAVA YGAAVQAAIL SGDKSENVQD LLLLDVTPLS
401 LGIETAGGVM TVLIKRNTTI PTKQTQTFTT YSDNQPGVLI QVYEGERAMT
451 KDNLLGKFE LTGIPPAPRG VPQIEVTFDI DANGILNVSA VDKSTGKENK
501 ITITNDKGR L SKEDIERMVQ EAEKYKAED KQDKVSSKN SLESYAFNMK
551 ATVEDEKLQG KINDEDKQKI LDKCNEIISW LDKNQTAKE EFEHQQKELE
601 KVCNPIITKL YQSAGGMPGG MPGGFPGGGA PPSGGASSGP TIEEVD

```

s7: gi 8393322; Protein disulfide isomerase A3 precursor (Disulfide isomerase ER-**60)**

```

1 MRFSCALLP GVALLLASAL LASASDVLEL TDENFESRVS DTGSAGMLLV
51 EFFAPWCGHC KRLAPEYEAA ATRLKGIVPL AKVDCTANTN TCNKYGVSGY
101 PTLKIFRDGE EAGAYDGPR ADGIVSHLKK QAGPASVPLR TEDEFKKFIS
151 DKDASVVGFF RDLFSDGHSE FLKAASNLRD NYRFAHTNVE SLVKEYDDNG
201 EGITIFRPLH LANKFEDKIV AYTEKKMTSG KIKKFIQESI FGLCPHMTED
251 NKDLIQGKDL LTAYYDVDEY KNTKGSNYWR NRVMMAKTF LDAGHKLNFA
301 VASRKTFSHE LSDFGLESTT GEIPVVAIRT AKGEKFVMQE EFSRDGKALE
351 RFLQEYFDGN LKRYLKSEPI PETNEGPVKV VVAESFDDIV NAEDKDVLEI
401 FYAPWCGHCK NLEPKYKELG EKLSKDPNIV IAKMDATAND VPSPYEVKGF
451 PTIYFSPANK KLTPKKYEGG RELNDLISYL QREATNPPII QEEKPKKKKK
501 AQEDL

```


s7a: gi 8393322; Protein disulfide isomerase A3 precursor (Disulfide isomerase ER-60)

```

1 MRFSC LALLP GVALLLASAL LASASDVLEL TDENFESRVS DTGSAGLMLV
51 EFFAPWCGHC KRLAPEYEAA ATRLKGIVPL AKVDCTANTN TCNKYGVSGY
101 PTLKIFRDGE EAGAYDGPRT ADGIVSHLKK QAGPASVPLR TEDEFKKFIS
151 DKDASVVGFF RDLFSDGHSE FLKAASNLRD NYRFAHTNVE SLVKEYDDNG
201 EGITIFRPLH LANKFEDKIV AYTEKKMTSG KIKKFIQESI FGLCPHMTED
251 NKDLIQGKDL LTAYYDVDYE KNTKGSNYWR NRVMMVAKTF LDAGHKLNFA
301 VASRKTFSHE LSDFGLESTT GEIPVVAIRT AKGEKFVMQE EFSRDGKALE
351 RFLQEYFDGN LKRYLKSEPI PETNEGPKV VVAESFDDIV NAEDKDVLIIE
401 FYAPWCGHCK NLEPKYKELG EKLSKDPNIV IAKMDATAND VPSPYEVKGF
451 PTIYFSPANK KLTPKKYEGG RELNDLISYL QREATNPPII QEEKPKKKKK
501 AQEDL

```

s20: gi 51036657; Prolyl 4-hydroxylase alpha-1 subunit

```

1 MIWGVLMGI LLPQCSAHPG FFTSIGQMTD LIHNEKDLVT SLKDYIKAE
51 DKLEQIKKWA EKLDRLTSTA TKDPEGFVGH PVNAFKLMKR LNTEWSELEN
101 LILKDMSDGF ISNLTIQRY FPNDEDQVGA AKALFRLQDT YNLDTNTISK
151 GNLPGVKHKS FLTAEDCFEL GKVAYTEADY YHTELWMEQA LMQLEEGEMS
201 TVDKVSVLDY LSYAVYQQGD LDKALLLTKK LLELDPEHQR ANGNLVYFEY
251 IMSKEKDANK SASGDQSDQK TTPKKKGIAV DYLPERQKYE MLCRGEIGKM
301 TPRRQKRLFC RYHDGNRNPK FILAPAKQED EWDKPRIIRF HDIISDAEIE
351 IVKDLAKPRL SRATVHDPET GKLTTAQYRV SKSAWLSGYE DPVVSRINMR
401 IQDLTGLDVS TAEELQVANY GVGGQYEPHF DFARKDEPDA FRELGTGNRI
451 ATWLFYMSDV SAGGATVFPE VGASVWPKKG TAVFWYNLFA SGEGDYSTRH
501 AACPVLVGNK WWSNKWLHER GQEFRRPCTL SELE

```

s8: gi 13592133; beta-actin

```

1 MDDDIAALVV DNGSAMCKAG FAGDDAPRAV FPSIVGRPRH QGVMVGMGQK
51 DSYVGDEAQS KRGILTLYKYP IEHGIVTNWD DMEKIWHHTF YNELRVAPEE
101 HPVLLTEAPL NPKANREKMT QIMFETFNTP AMYVAIQAVL SLYASGRTTG
151 IVMDSGDGVV HTVPIYEGYA LPHAILRLDL AGRDLTDYLM KILTERGYSF
201 TTTAEREIVR DIKEKLCYVA LDFEQEMATA ASSSSLEKSY ELPDGQVITI
251 GNERFRCPEA LFQPSFLGME SCGIHETTFN SIMKCDVDIR KDLYANTVLS
301 GGTTMYPGIA DRMQKEITAL APSTMKIKII APPERKYSVW IGGSILASLS
351 TFQQMWISKQ EYDESGPSIV HRKCF

```

s8a: gi 13592133; beta-actin

```

1 MDDDIAALVV DNGSAMCKAG FAGDDAPRAV FPSIVGRPRH QGVMVGMGQK
51 DSYVGDEAQS KRGILTLYKYP IEHGIVTNWD DMEKIWHHTF YNELRVAPEE
101 HPVLLTEAPL NPKANREKMT QIMFETFNTP AMYVAIQAVL SLYASGRTTG
151 IVMDSGDGVV HTVPIYEGYA LPHAILRLDL AGRDLTDYLM KILTERGYSF
201 TTTAEREIVR DIKEKLCYVA LDFEQEMATA ASSSSLEKSY ELPDGQVITI
251 GNERFRCPEA LFQPSFLGME SCGIHETTFN SIMKCDVDIR KDLYANTVLS
301 GGTTMYPGIA DRMQKEITAL APSTMKIKII APPERKYSVW IGGSILASLS
351 TFQQMWISKQ EYDESGPSIV HRKCF

```

s9: gi 13592133; beta-actin

```

1  PRAVFPSIVG RSRHQGVMMVG MGQKDSYVGD EAQSKRGILT LKYPIEHGIV
51  TNWDDMEKIW HHTFYNELRV APEEHPVLLT EAPLNPKANR EKMTQIMFET
101 FNTPAMYVAI QAVLSLYASG RTTGIVMDSG DGVTHTVPIY EGYALPHAIL
151 RLDLAGRDLT DYLMKILTER GYSFTTTAER EIVRDIKEKL CYVALDFEQE
201 MATAASSSSL EKSYELPDGQ VITIGNERFR CPEALFQPSF LGMESCGIHE
251 TTFNSIMKCD VDIRKDLYAN TVLSGGTTMY PGIADRMQKE ITALAPSTMK
301 IKIIAPPERK YSVWIGGSIL ASLSTFQQMW ISKQEYDESG PSIVHRKCF

```

s10: gi 49868; putative beta-actin (aa 27-375)

```

1  PRAVFPSIVG RSRHQGVMMVG MGQKDSYVGD EAQSKRGILT LKYPIEHGIV
51  TNWDDMEKIW HHTFYNELRV APEEHPVLLT EAPLNPKANR EKMTQIMFET
101 FNTPAMYVAI QAVLSLYASG RTTGIVMDSG DGVTHTVPIY EGYALPHAIL
151 RLDLAGRDLT DYLMKILTER GYSFTTTAER EIVRDIKEKL CYVALDFEQE
201 MATAASSSSL EKSYELPDGQ VITIGNERFR CPEALFQPSF LGMESCGIHE
251 TTFNSIMKCD VDIRKDLYAN TVLSGGTTMY PGIADRMQKE ITALAPSTMK
301 IKIIAPPERK YSVWIGGSIL ASLSTFQQMW ISKQEYDESG PSIVHRKCF

```

s18: gi 14389299; Vimentin

```

1  MSTRSVSSSS YRRMFGGSGT SSRPSSNRSY VTTSTRTYSL GSALRPSTSR
51  SLYSSSPGGA YVTRSSAVRL RSSMPGVRL QDSVDFSLAD AINTEFKNTR
101 TNEKVELQEL NDRFANYIDK VRFLEQQNKI LLAELEQLKG QGKSRLGDLY
151 EEEMRELRRQ VDQLTNDKAR VEVERDNLA E DIMRLREKLQ E EMLQREEAE
201 STLQSFRQDV DNASLARLDL ERKVESLQEE IAFLLKHLDE EIQELQAQIQ
251 EQHVQIDVDV SKPDLTAALR DVRQQYESVA AKNLQEAEEW YKSKFADLSE
301 AANRNNDALR QAKQESNEYR RQVQSLTCEV DALKGTNESL ERQRMREEN
351 FALEAANYQD TIGRLQDEIQ NMKEEMARHL REYQDLLNVK MALDIEIATY
401 RKLLEGEESR ISLPLPNFSS LNLRETNLES LPLVDTHSKR TLLIKTVETR
451 DGQVINETSQ HHDDLE

```

s18: gi11560133; Tubulin alpha

```

1  MRECISIHVG QAGVQIGNAC WELYCLEHGI QPDGQMPSDK TIGGGDDSFN
51  TFFSETGAGK HVPRAVFVDL EPTVIDEVRT GTYRQLFHPE QLITGKEDAA
101 NNYARGHYTI GKEIIDLVLD RIRKLADQCT GLQGFLVFHS FGGGTGSGFT
151 SLLMERLSVD YGKKSKEFS IYPAPQVSTA VVEPYNSILT THTTLEHSDC
201 AFMVDNEAIY DICRRNLDIE RPTYTNLNR L IGQIVSSITA SLRFDGALNV
251 DLTEFQTNLV PYPRIHFPLA TYAPVISA EK AYHEQLSVAE ITNACFEPAN
301 QMVKCDPRHG KYMACCLLYR GDVVPKDVNA AIATIKTKRT IQFVDWCPTG
351 FKVGINYPQP TVVPGGDLAK VQRAVCMLSN TTAIAEAWAR LDHKFDLMYA
401 KRAFVHWYVG EGMEEGEFSE AREDMAALEK DYEEVGVDSV EYEGEEEGEE
451 Y

```


s19: gi 14389299; Vimentin

```

1  MSTRSVSSSS YRRMFGGSGT SSRPSSNRSY VTTSTRTYSL GSALRPSTSR
51 SLYSSSPGGGA YVTRSSAVRL RSSMPGVRL QDSVDFSLAD AINTEFKNTR
101 TNEKVELQEL NDRFANYIDK VRFLEQQNKI LLAELEQLKG QGKSRLGDLY
151 EEEMRELRRQ VDQLTNDKAR VEVERDNLAE DIMRLREKLQ EEMLRQEEAE
201 STLQSFQRDV DNASLARLDL ERKVESLQEE IAFLKKLHDE EIQELQAQIQ
251 EQHVQIDVDV SKPDLTAALR DVRQYESVA AKNLQEAEW YKSKFADLSE
301 AANRNNDALR QAKQESNEYR RQVQSLTCEV DALKGTNESL ERQMREMEEN
351 FALEAANYQD TIGRLQDEIQ NMKEEMARHL REYQDLLNVK MALDIEIATY
401 RKLEGEESR ISLPLNFSS LNLRETNLES LPLVDTHSKR TLLIKTVETR
451 DGQVINETSQ HHDDLE

```

s15: gi 6978809; Enolase-1, alpha

```

1  MSILKIHARE IFDSRGNPTV EVDLYTAKGL FRAAVPSGAS TGIYEALELR
51 DNDKTRFMGK GVSKAVEHIN KTIAPALVSK KLNVVEQEKI DQLMIEMDGT
101 ENKSKFGANA ILGVSLAVCK AGAVEKGVPL YRHIADLAGN PEVILPVPFAP
151 NNINGGSHAG NKLAMQEFMI LPVGASSFRE AMRIGAEVYH NLKNVIKEKY
201 GKDATNVGDE GGFAPNILEN KEALELLKSA IAKAGYTDQV VIGMDVAASE
251 FYRAGKYDLD FKSPDDASRY ITPDQLADLY KSFIKDYPVV SIEDPFDQDD
301 WDAWQKFTAT AGIQVVGDDL TVTNPKRIAK AAGEKSCNCL LLKVNQIGSV
351 TESLQACKLA QSNWGVVMVS HRSGETEDTF IADLVVGLCT GQIKTGAPCR
401 SERLAKYNQI LRIEEEELGSK AKFAGRSFRN PLAK

```

s16: gi 6978809; Enolase-1, alpha

```

1  MSILKIHARE IFDSRGNPTV EVDLYTAKGL FRAAVPSGAS TGIYEALELR
51 DNDKTRFMGK GVSKAVEHIN KTIAPALVSK KLNVVEQEKI DQLMIEMDGT
101 ENKSKFGANA ILGVSLAVCK AGAVEKGVPL YRHIADLAGN PEVILPVPFAP
151 NNINGGSHAG NKLAMQEFMI LPVGASSFRE AMRIGAEVYH NLKNVIKEKY
201 GKDATNVGDE GGFAPNILEN KEALELLKSA IAKAGYTDQV VIGMDVAASE
251 FYRAGKYDLD FKSPDDASRY ITPDQLADLY KSFIKDYPVV SIEDPFDQDD
301 WDAWQKFTAT AGIQVVGDDL TVTNPKRIAK AAGEKSCNCL LLKVNQIGSV
351 TESLQACKLA QSNWGVVMVS HRSGETEDTF IADLVVGLCT GQIKTGAPCR
401 SERLAKYNQI LRIEEEELGSK AKFAGRSFRN PLAK

```

s17: gi 6978809; Enolase-1, alpha

```

1  MSILKIHARE IFDSRGNPTV EVDLYTAKGL FRAAVPSGAS TGIYEALELR
51 DNDKTRFMGK GVSKAVEHIN KTIAPALVSK KLNVVEQEKI DQLMIEMDGT
101 ENKSKFGANA ILGVSLAVCK AGAVEKGVPL YRHIADLAGN PEVILPVPFAP
151 NNINGGSHAG NKLAMQEFMI LPVGASSFRE AMRIGAEVYH NLKNVIKEKY
201 GKDATNVGDE GGFAPNILEN KEALELLKSA IAKAGYTDQV VIGMDVAASE
251 FYRAGKYDLD FKSPDDASRY ITPDQLADLY KSFIKDYPVV SIEDPFDQDD
301 WDAWQKFTAT AGIQVVGDDL TVTNPKRIAK AAGEKSCNCL LLKVNQIGSV
351 TESLQACKLA QSNWGVVMVS HRSGETEDTF IADLVVGLCT GQIKTGAPCR
401 SERLAKYNQI LRIEEEELGSK AKFAGRSFRN PLAK

```

s22: gi 26023949; Enolase-2, gamma

```

1  MSIQKIWARE  ILDSRGNPTV  EVDLHTAKGL  FRAAVPSGAS  TGIYEALELR
51  DGDQORYLGK  GVLKAVDHIN  STIAPALISS  GLSVVEQEKL  DNLMLELDGT
101 ENKSKFGANA  ILGVSLAVCK  AGAAEKDLPL  YRHIAQLAGN  SDLILPVPAF
151 NVINGGSHAG  NKLAMQEFMI  LPVGAESFRD  AMRLGAEVYH  TLKGVIKDKY
201 GKDATNVGDE  GGFAPNILEN  SEALELVKEA  IDKAGYTEKM  VIGMDVAASE
251 FYRDGKYDLD  FKSPADPSRC  ITGDLGALY  QDFVRNYPVV  SIEDPFDQDD
301 WAAWSKFTAN  VGIQIVGDDL  TVTNPKRIER  AVEEKACNCL  LLKVNQIGSV
351 TEAIQACKLA  QENGWGMVS  HRSGETEDTF  IADLVVGLCT  GQIKTGAPCR
401 SERLAKYNQL  MRIEELGEE  ARFAGHNFRN  PSVL

```

s12: gi 730681; 40S ribosomal protein SA

```

1  MSGGLDVLQM  KEEDVLKFLA  AGTHLGGTNL  DFQMEQYIYK  RKSDGIYIIN
51  LKRTWEKLLL  AARAIVAIEN  PADVSVISSR  NTGQRAVLKF  AAATGATPIA
101 GRFTPGTFTN  QIQAAFREPR  LLVVTDPRAD  HQPLTEASYV  NLPTIALCNT
151 DSPLRYVDIA  IPCNNKGAHS  VGLMWWMLAR  EVLRMRGTIS  REHPWEVMPD
201 LYFYRDPEEI  EKEEQAAAEK  AVTKEEFQGE  WTAPAPEFTA  AQPEVADWSE
251 GVQVPSVPIQ  QFPTEDWSAQ  PATEDWSAAP  TAQATEWVGA  TTEWS

```


s14: gi 14286100; Plasma membrane calcium transporting ATP-ase (PMCA2)

There are 12 splice isoforms of this protein. The “XB” splice isoform was identified with MALDI-TOF and Swis Prot database search. The “XB” splice isoform does not contain the sequence 303-333 (highlighted in yellow) and is the only splice isoform that has Arg¹¹⁴² (highlighted in black).

```

 1 MGDMTNSDFY SKNQRESSH GGEFGCSMEE LRSLMELRGT EAVVIKETY
51 GDTESICRRL KTSPVEGLPG TAPDLEKRKQ IFGQNFIPPK KPKTFLQLVW
101 EALQDVTLII LEIAAIIISLG LSFYHPPGES NEGCATAQGG AEDEGEAEAG
151 WIEGAAILLS VICVVLVTAF NDWSKEKQFR GLQSRIEQEQ KFTVVRAGQV
201 VQIPVAEIVV GDIAQIKYGD LLPADGLFIQ GNDLKIDESS LTGESDQVRK
251 SVDKDPMLLS GTHVMEGSGR MVVTAVGVNS QTGIIFTLLG AGGEEEEKKD
301 KKGVKKGDGL QLPAADGAAP ANAAGSANAS LVNGKMQDGS ADSSQSKAKQ
351 QDGAAAMEMQ PLKSAEGGDA DDKKKANMHK KEKSVLQGKL TKLAVQIGKA
401 GLVMSAITVI ILVLYFTVDT FVVNKKPWLT ECTPVYVQYF VKFFIIGTV
451 LVVAVPEGLP LAVTISLAYS VKKMMKDNNL VRHLDACETM GNATAICSDK
501 TGTLTTNRMT VVQAYVGDVH YKEIPDPSSI NAKTLELLVN AIAINSAYTT
551 KILPPEKEGA LPRQVGNKTE CGLLGFVLDL RQDYEPVRSQ MPEEKLYKVY
601 TFNSVRKSMS TVIKMPDESF RMYSKGASEI VLKKCKILS GAGEPRVFRP
651 RDRDEMVKKV IEPMACDGLR TICVAYRDFP SSPEPDWDNE NDILNELTCI
701 CVVGIEDPVR PEVPEAIRKC QRAGITVRMV TGDNINTARA IAIKCGIIHP
751 GEDFLCLEGK EFNRRIRNEK GEIEQERIDK IWPKLRVLAR SSPTDKHTLV
801 KGIIDSTHTE QRQVAVTGD GTNDGPALKK ADVGFAMGIA GTDVAKEASD
851 IILTDDNFSS IVKAVMWGRN VYDSISKFLQ FQLTVNVVAV IVAFTGACIT
901 QDSPLKAVQM LWVNLIMDTF ASLALATEPP TETLLLRKPY GRNKPLISRT
951 MMKNILGHAV YQLTLIFTLL FVGEKMFQID SGRNAPLHSP PSEHYTIIFN
1001 TFVMMQLFNE INARKIHGER NVFDGIFRNP IFCTIVLGTF AIQIVIVQFG
1051 GKPFSCSPLQ LDQWMWCIFI GLGELVWGQV IATIPTSRLK FLKEAGRLTQ
1101 KEEIPEEELN EDVEEIDHAE RELRRGQILW FRGLNRIQTQ IVVKAFRSS
1151 LYEGLEKPES RTSIHNFMAH PEFRIEDSQP HIPLIDDTDL EEDAALKQNS
1201 SPPSSLNKNN SAIDSGINLT TDTSKSATSS SPGSPIHSLE TSL

```

s6: gi 21759130; RhoGDI-1

```

 1 MAEQEPTAEQ LAQIAAENEE DEHSVNYKPP AQKSIQEIQE LDKDDESLRK
51 YKEALLGRVA VSADPNVPNV IVTRLTLVCS TAPGPLELDL TGDLESFKKQ
101 SFVLKEGVEY RIKISFRVNR EIVSGMKYIQ HTYRKGVKID KTDYMVGSYG
151 PRAEYEFLT PMEEAPKGML ARGSYNIKSR FTDDDKTDHL SWEWNLTIKK
201 EWKD

```

s13: gi 124902; Interferon regulatory factor 1 (IRF-1)

```
1  MPITRMRRP WLEMQINSNQ IPGLSWINKE EMIFQIPWKH AALHGWDINK
51  DACLFRSWAI HTGRYKAGEK EPDPKTWKAN FRCAMNSLPD IEEVKDQSRN
101 KGSSAVRVYR MLPPLTKNQR KERKSKSSRD TSKTKRKLC GDSSPDTLSD
151 GLSSSTLPDD HSSYTAQGYL GQDLMDRDI TPALSPCVVS SSLSEWHMQM
201 DIMPDSTDL YNLQVSPMPS TSEAATDEDE EGKLPEDIMK LFEQSEWQPT
251 HVDGKGYLLN EPGAQLSTVY GDFSCKEEPE IDSPGGDIEI GIQRVFTEMK
301 NMDPVMWMDT LLGNSTRPPS IQAIPCAP
```


C. MALDI-TOF mass spectra

Representative MALDI-TOF mass spectra are shown for 2 proteins from each table (Table 3-1 and Table 5-1).

Table 3-1, s15 Tropomyosin 2

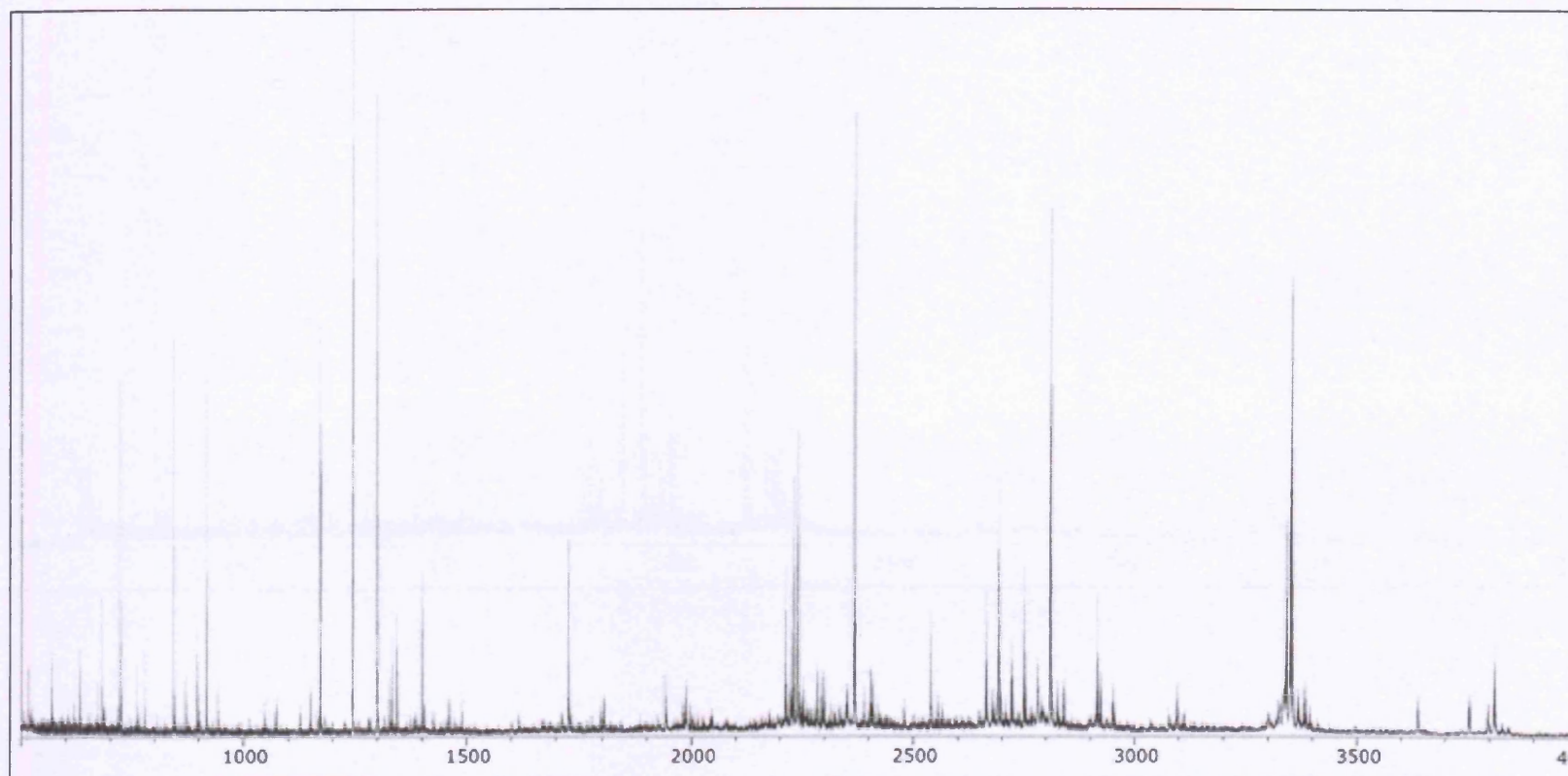


Table 3-1, s5 Heat shock 70 kDa protein 5 (GRP78)

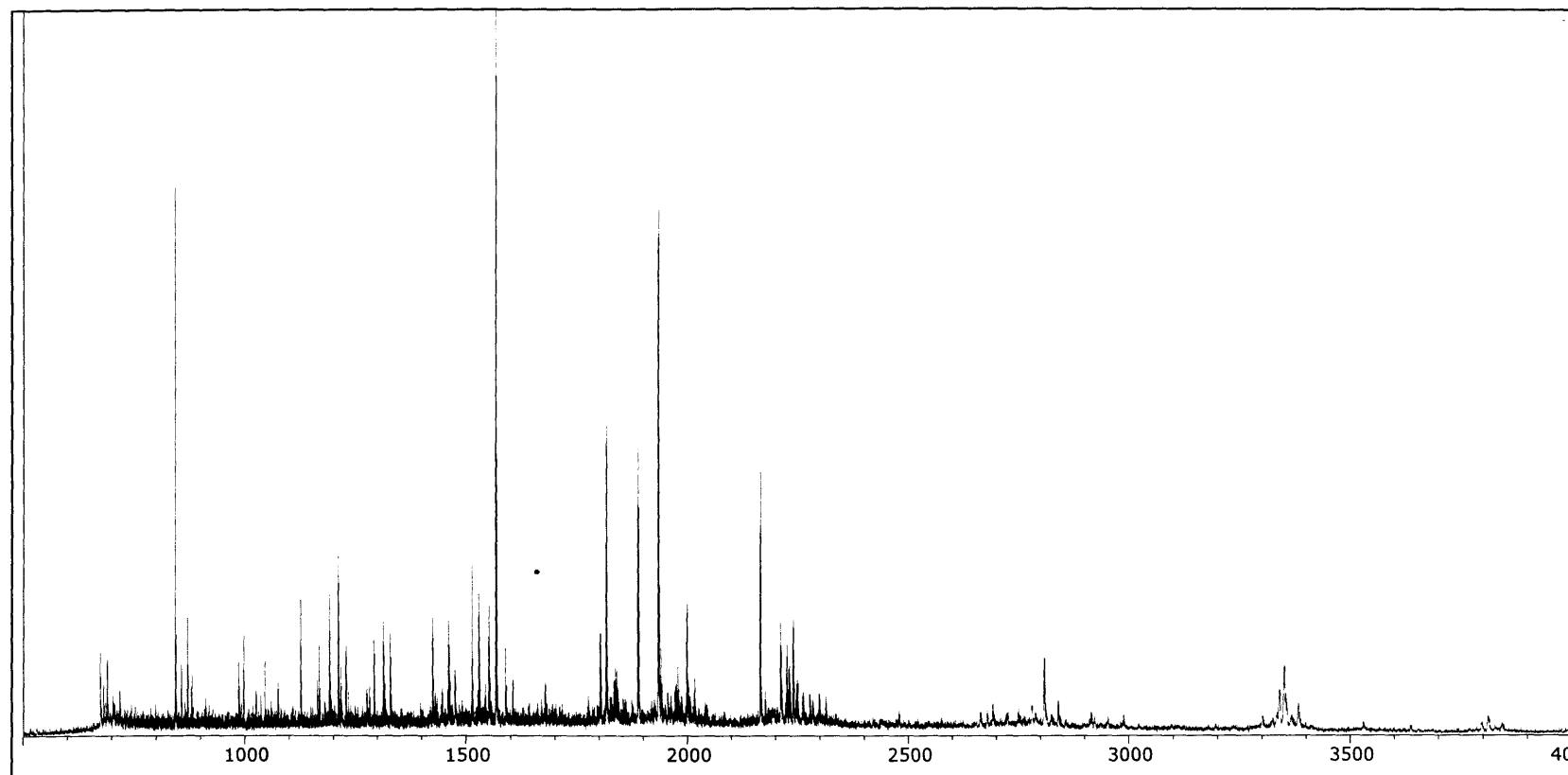


Table 5-1, s7 ER-60 protease (GRP58)

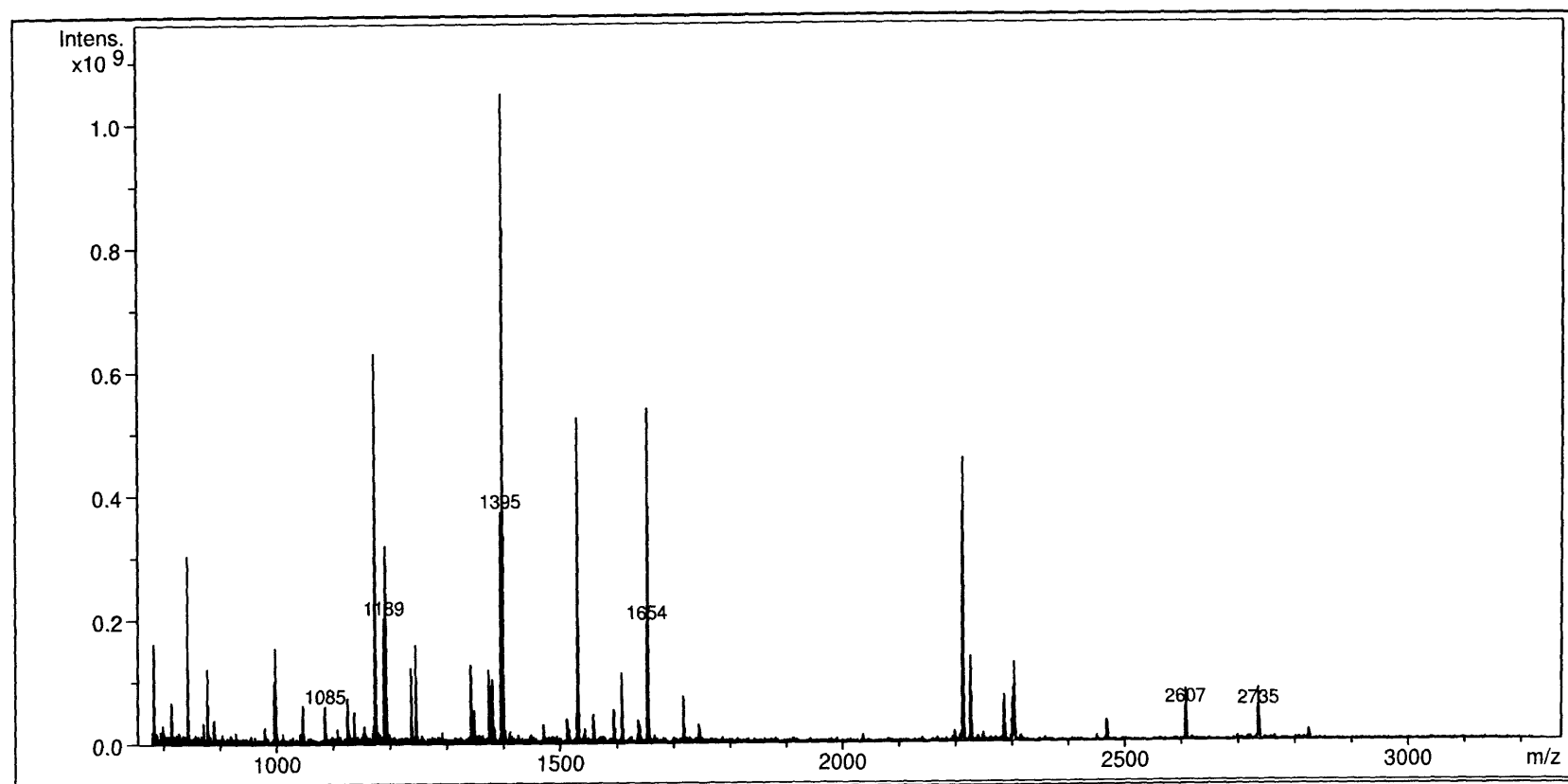
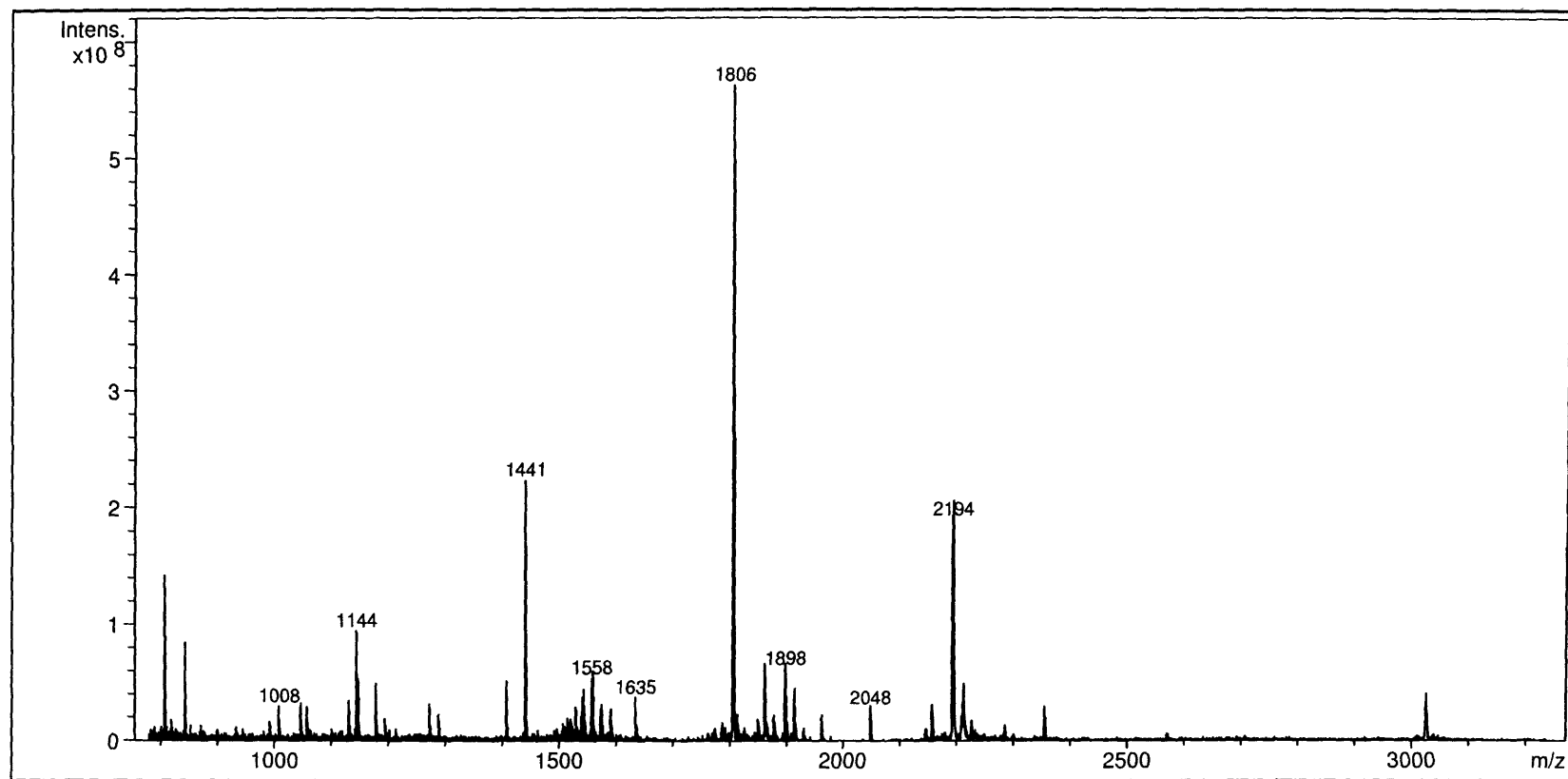


Table 5-1, s16 Enolase-1



REFERENCES

Abassi Z, Gurbanov K, Rubinstein I, Better OS, Hoffman A, Winaver J (1998). Regulation of intrarenal blood flow in experimental heart failure: role of endothelin and nitric oxide. *Am J Physiol* 274:F766-F774.

Abdel-Latif AA, Husain S, Yousufzai SY (2000). Role of protein kinase C alpha and mitogen-activated protein kinases in endothelin-1-stimulation of cytosolic phospholipase A2 in iris sphincter smooth muscle. *J Cardiovasc Pharmacol* 36:S117-S119.

Abo A, Webb MR, Grogan A, Segal AW (1994). Activation of NADPH oxidase involves the dissociation of p21rac from its inhibitory GDP/GTP exchange protein (rhoGDI) followed by its translocation to the plasma membrane. *Biochem J* 298 Pt 3:585-591.

Adam LP, Milio L, Brengle B, Hathaway DR (1990). Myosin light chain and caldesmon phosphorylation in arterial muscle stimulated with endothelin-1. *J Mol Cell Cardiol* 22:1017-1023.

Adamo HP, Penniston JT (1992). New Ca²⁺ pump isoforms generated by alternative splicing of rPMCA2 mRNA. *Biochem J* 283 (Pt 2):355-359.

Alberts GF, Peifley KA, Johns A, Kleha JF, Winkles JA (1994). Constitutive endothelin-1 overexpression promotes smooth muscle cell proliferation via an external autocrine loop. *J Biol Chem* 269:10112-10118.

Ambar I, Kloog Y, Kochva E, Wollberg Z, Bdolah A, Oron U, Sokolovsky M (1988). Characterization and localization of a novel neuroreceptor for the peptide sarafotoxin. *Biochem Biophys Res Commun* 157:1104-1110.

Ames RS, Sarau HM, Chambers JK, Willette RN, Aiyar NV, Romanic AM, Louden CS, Foley JJ, Sauermelch CF, Coatney RW, Ao Z, Disa J, Holmes SD, Stadel JM, Martin JD, Liu WS, Glover GI, Wilson S, McNulty DE, Ellis CE, Elshourbagy NA, Shabon U, Trill JJ, Hay DW, Ohlstein EH, Bergsma DJ, Douglas SA (1999). Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature* 401:282-286.

Andersen JS, Wilkinson CJ, Mayor T, Mortensen P, Nigg EA, Mann M (2003). Proteomic characterization of the human centrosome by protein correlation profiling. *Nature* 426:570-574.

Anderson KE, Coadwell J, Stephens LR, Hawkins PT (1998). Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B. *Curr Biol* 8:684-691.

Anderson L, Seilhamer J (1997). A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* 18:533-537.

Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* 348:730-732.

Aramori I, Nakanishi S (1992). Coupling of two endothelin receptor subtypes to differing signal transduction in transfected Chinese hamster ovary cells. *J Biol Chem* 267:12468-12474.

Bagnato A, Tecce R, Di C, V, Catt KJ (1997). Activation of mitogenic signaling by endothelin 1 in ovarian carcinoma cells. *Cancer Res* 57:1306-1311.

Bagnato A, Spinella F (2003). Emerging role of endothelin-1 in tumor angiogenesis. *Trends Endocrinol Metab* 14:44-50.

- Balasubramanian S, Teissere JA, Raju DV, Hall RA (2004). Hetero-oligomerization between GABAA and GABAB receptors regulates GABAB receptor trafficking. *J Biol Chem* 279:18840-18850.
- Ball RL, Albrecht T, Thompson WC, James O, Carney DH (1992). Thrombin, epidermal growth factor, and phorbol myristate acetate stimulate tubulin polymerization in quiescent cells: a potential link to mitogenesis. *Cell Motil Cytoskeleton* 23:265-278.
- Baynash AG, Hosoda K, Giaid A, Richardson JA, Emoto N, Hammer RE, Yanagisawa M (1994). Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. *Cell* 79:1277-1285.
- Benigni A, Remuzzi G (1999). Endothelin antagonists. *Lancet* 353:133-138.
- Biondi RM (2004). Phosphoinositide-dependent protein kinase 1, a sensor of protein conformation. *Trends Biochem Sci* 29:136-142.
- Bishop AL, Hall A (2000). Rho GTPases and their effector proteins. *Biochem J* 348 Pt 2:241-255.
- Bisotto S, Fixman ED (2001). Src-family tyrosine kinases, phosphoinositide 3-kinase and Gab1 regulate extracellular signal-regulated kinase 1 activation induced by the type A endothelin-1 G-protein-coupled receptor. *Biochem J* 360:77-85.
- Blagoev B, Kratchmarova I, Ong SE, Nielsen M, Foster LJ, Mann M (2003). A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling. *Nat Biotechnol* 21:315-318.
- Blenis J, Spivack JG, Erikson RL (1984). Phorbol ester, serum, and rous sarcoma virus transforming gene product induce similar phosphorylations of ribosomal protein S6. *Proc Natl Acad Sci U S A* 81:6408-6412.

- Bobik A, Grooms A, Millar JA, Mitchell A, Grinpukel S (1990). Growth factor activity of endothelin on vascular smooth muscle. *Am J Physiol* 258:C408-C415.
- Bogoyevitch MA, Ketterman AJ, Sugden PH (1995). Cellular stresses differentially activate c-Jun N-terminal protein kinases and extracellular signal-regulated protein kinases in cultured ventricular myocytes. *J Biol Chem* 270:29710-29717.
- Booz GW, Day JN, Speth R, Baker KM (2002). Cytokine G-protein signaling crosstalk in cardiomyocytes: attenuation of Jak-STAT activation by endothelin-1. *Mol Cell Biochem* 240:39-46.
- Bourmeyster N, Vignais PV (1996). Phosphorylation of Rho GDI stabilizes the Rho A-Rho GDI complex in neutrophil cytosol. *Biochem Biophys Res Commun* 218:54-60.
- Bowles J, Schepers G, Koopman P (2000). Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. *Dev Biol* 227:239-255.
- Boyle WJ, Smeal T, Defize LH, Angel P, Woodgett JR, Karin M, Hunter T (1991). Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. *Cell* 64:573-584.
- Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC (2003). Regulation of apoptosis by endoplasmic reticulum pathways. *Oncogene* 22:8608-8618.
- Bremnes T, Paasche JD, Mehluum A, Sandberg C, Bremnes B, Attramadal H (2000). Regulation and intracellular trafficking pathways of the endothelin receptors. *J Biol Chem* 275:17596-17604.
- Bruno CM, Neri S, Sciacca C, Caruso L (2000). Plasma endothelin-1 levels in liver cirrhosis. *Int J Clin Lab Res* 30:169-172.

- Bruno CM, Meli S, Marcinno M, Ierna D, Sciacca C, Neri S (2002). Plasma endothelin-1 levels and albumin excretion rate in normotensive, microalbuminuric type 2 diabetic patients. *J Biol Regul Homeost Agents* 16:114-117.
- Buto S, Tagliabue E, Ardini E, Magnifico A, Ghirelli C, van den BF, Castronovo V, Colnaghi MI, Sobel ME, Menard S (1998). Formation of the 67-kDa laminin receptor by acylation of the precursor. *J Cell Biochem* 69:244-251.
- Calalb MB, Polte TR, Hanks SK (1995). Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Mol Cell Biol* 15:954-963.
- Carlino A, Toledo H, Vidal V, Redfield B, Strassman J, Abdel-Ghany M, Racker E, Weissbach H, Brot N (1994). BiP is a substrate for src kinase in vitro. *Biochem Biophys Res Commun* 201:1548-1553.
- Carter DA (1997). Modulation of cellular AP-1 DNA binding activity by heat shock proteins. *FEBS Lett* 416:81-85.
- Cartwright CA, Mamajiwalla S, Skolnick SA, Eckhart W, Burgess DR (1993). Intestinal crypt cells contain higher levels of cytoskeletal-associated pp60c-src protein tyrosine kinase activity than do differentiated enterocytes. *Oncogene* 8:1033-1039.
- Cattaruzza M, Dimigen C, Ehrenreich H, Hecker M (2000). Stretch-induced endothelin B receptor-mediated apoptosis in vascular smooth muscle cells. *FASEB J* 14:991-998.
- Caverzasio J, Palmer G, Suzuki A, Bonjour JP (2000). Evidence for the involvement of two pathways in activation of extracellular signal-regulated kinase (Erk) and cell proliferation by Gi and Gq protein-coupled receptors in osteoblast-like cells. *J Bone Miner Res* 15:1697-1706.

- Cazaubon S, Parker PJ, Strosberg AD, Couraud PO (1993). Endothelins stimulate tyrosine phosphorylation and activity of p42/mitogen-activated protein kinase in astrocytes. *Biochem J* 293 (Pt 2):381-386.
- Cazaubon S, Chaverot N, Romero IA, Girault JA, Adamson P, Strosberg AD, Couraud PO (1997). Growth factor activity of endothelin-1 in primary astrocytes mediated by adhesion-dependent and -independent pathways. *J Neurosci* 17:6203-6212.
- Celli CM, Jaiswal AK (2003). Role of GRP58 in mitomycin C-induced DNA cross-linking. *Cancer Res* 63:6016-6025.
- Chackalaparampil I, Bagrodia S, Shalloway D (1994). Tyrosine dephosphorylation of pp60c-src is stimulated by a serine/threonine phosphatase inhibitor. *Oncogene* 9:1947-1955.
- Chaga GS (2001). Twenty-five years of immobilized metal ion affinity chromatography: past, present and future. *J Biochem Biophys Methods* 49:313-334.
- Chapman RS, Duff EK, Lourenco PC, Tonner E, Flint DJ, Clarke AR, Watson CJ (2000). A novel role for IRF-1 as a suppressor of apoptosis. *Oncogene* 19:6386-6391.
- Chisholm LJ, Agrawal DK, Pearson TJ, Edwards JD (1996). Endothelin-1 induces tyrosine phosphorylation in human blood monocytes. *Mol Cell Biochem* 159:33-38.
- Choudhary S, De BP, Banerjee AK (2000). Specific phosphorylated forms of glyceraldehyde 3-phosphate dehydrogenase associate with human parainfluenza virus type 3 and inhibit viral transcription in vitro. *J Virol* 74:3634-3641.
- Chrzanowska-Wodnicka M, Burridge K (1996). Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J Cell Biol* 133:1403-1415.

- Chu A, Matusiewicz N, Stochaj U (2001). Heat-induced nuclear accumulation of hsc70s is regulated by phosphorylation and inhibited in confluent cells. *FASEB J* 15:1478-1480.
- Chun M, Liyanage UK, Lisanti MP, Lodish HF (1994). Signal transduction of a G protein-coupled receptor in caveolae: colocalization of endothelin and its receptor with caveolin. *Proc Natl Acad Sci U S A* 91:11728-11732.
- Clerk A, Michael A, Sugden PH (1998). Stimulation of the p38 mitogen-activated protein kinase pathway in neonatal rat ventricular myocytes by the G protein-coupled receptor agonists, endothelin-1 and phenylephrine: a role in cardiac myocyte hypertrophy? *J Cell Biol* 142:523-535.
- Clouthier DE, Hosoda K, Richardson JA, Williams SC, Yanagisawa H, Kuwaki T, Kumada M, Hammer RE, Yanagisawa M (1998). Cranial and cardiac neural crest defects in endothelin-A receptor-deficient mice. *Development* 125:813-824.
- Cohen GB, Ren R, Baltimore D (1995). Modular binding domains in signal transduction proteins. *Cell* 80:237-248.
- Comolli R, Alberti P, Frigerio M (1988). Activation of a ribosomal protein S6 kinase during the 4-dimethylaminoazobenzene-induced rat hepatocarcinogenesis. *Cancer Lett* 41:281-285.
- Constantin B, Meerschaert K, Vandekerckhove J, Gettemans J (1998). Disruption of the actin cytoskeleton of mammalian cells by the capping complex actin-fragmin is inhibited by actin phosphorylation and regulated by Ca²⁺ ions. *J Cell Sci* 111 (Pt 12):1695-1706.
- Cooper JA, Reiss NA, Schwartz RJ, Hunter T (1983). Three glycolytic enzymes are phosphorylated at tyrosine in cells transformed by Rous sarcoma virus. *Nature* 302:218-223.

- Cooper JA, Howell B (1993). The when and how of Src regulation. *Cell* 73:1051-1054.
- Coss MC, Winterstein D, Sowder RC, Simek SL (1995). Molecular cloning, DNA sequence analysis, and biochemical characterization of a novel 65-kDa FK506-binding protein (FKBP65). *J Biol Chem* 270:29336-29341.
- Cramer H, Muller-Esterl W, Schroeder C (1997). Subtype-specific desensitization of human endothelin ETA and ETB receptors reflects differential receptor phosphorylation. *Biochemistry* 36:13325-13332.
- Crespo P, Xu N, Simonds WF, Gutkind JS (1994). Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. *Nature* 369:418-420.
- Cruz A, Parnot C, Ribatti D, Corvol P, Gasc JM (2001). Endothelin-1, a regulator of angiogenesis in the chick chorioallantoic membrane. *J Vasc Res* 38:536-545.
- Dagassan PH, Breu V, Clozel M, Kunzli A, Vogt P, Turina M, Kiowski W, Clozel JP (1996). Up-regulation of endothelin-B receptors in atherosclerotic human coronary arteries. *J Cardiovasc Pharmacol* 27:147-153.
- Damon DH (1998). Postganglionic sympathetic neurons express endothelin. *Am J Physiol* 274:R873-R878.
- Daub H, Weiss FU, Wallasch C, Ullrich A (1996). Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* 379:557-560.
- Davenport AP (2002). International Union of Pharmacology. XXIX. Update on endothelin receptor nomenclature. *Pharmacol Rev* 54:219-226.
- Davis EC, Broekelmann TJ, Ozawa Y, Mecham RP (1998). Identification of tropoelastin as a ligand for the 65-kD FK506-binding protein, FKBP65, in the secretory pathway. *J Cell Biol* 140:295-303.

de Nucci G, Thomas R, D'Orleans-Juste P, Antunes E, Walder C, Warner TD, Vane JR (1988). Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc Natl Acad Sci U S A* 85:9797-9800.

Dean WL, Chen D, Brandt PC, Vanaman TC (1997). Regulation of platelet plasma membrane Ca^{2+} -ATPase by cAMP-dependent and tyrosine phosphorylation. *J Biol Chem* 272:15113-15119.

Del Bufalo D, Di C, V, Biroccio A, Varmi M, Salani D, Rosano L, Triscioglio D, Spinella F, Bagnato A (2002). Endothelin-1 protects ovarian carcinoma cells against paclitaxel-induced apoptosis: requirement for Akt activation. *Mol Pharmacol* 61:524-532.

Donella-Deana A, James P, Staudenmann W, Cesaro L, Marin O, Brunati AM, Ruzzene M, Pinna LA (1996). Isolation from spleen of a 57-kDa protein substrate of the tyrosine kinase Lyn. Identification as a protein related to protein disulfide-isomerase and localisation of the phosphorylation sites. *Eur J Biochem* 235:18-25.

Douglas SA, Beck GR, Jr., Elliott JD, Ohlstein EH (1995). Pharmacological evidence for the presence of three distinct functional endothelin receptor subtypes in the rabbit lateral saphenous vein. *Br J Pharmacol* 114:1529-1540.

Douglas SA, Ohlstein EH (1997). Signal transduction mechanisms mediating the vascular actions of endothelin. *J Vasc Res* 34:152-164.

Drimal J, Drimal J, Jr., Drimal D (2000). Enhanced endothelin ET(B) receptor down-regulation in human tumor cells. *Eur J Pharmacol* 396:19-22.

Dupuis J, Rouleau JL, Cernacek P (1998). Reduced Pulmonary Clearance of Endothelin-1 Contributes to the Increase of Circulating Levels in Heart Failure Secondary to Myocardial Infarction. *Circulation* 98:1684-1687.

- Endoh M, Fujita S, Yang HT, Talukder MA, Maruya J, Norota I (1998). Endothelin: receptor subtypes, signal transduction, regulation of Ca²⁺ transients and contractility in rabbit ventricular myocardium. *Life Sci* 62:1485-1489.
- Eriksson JE, He T, Trejo-Skalli AV, Harmala-Brasken AS, Hellman J, Chou YH, Goldman RD (2004). Specific in vivo phosphorylation sites determine the assembly dynamics of vimentin intermediate filaments. *J Cell Sci* 117:919-932.
- Eude I, Paris B, Cabrol D, Ferre F, Breuiller-Fouche M (2000). Selective protein kinase C isoforms are involved in endothelin-1-induced human uterine contraction at the end of pregnancy. *Biol Reprod* 63:1567-1573.
- Faruki S, Geahlen RL, Asai DJ (2000). Syk-dependent phosphorylation of microtubules in activated B-lymphocytes. *J Cell Sci* 113 (Pt 14):2557-2565.
- Ficarro S, Chertihin O, Westbrook VA, White F, Jayes F, Kalab P, Marto JA, Shabanowitz J, Herr JC, Hunt DF, Visconti PE (2003). Phosphoproteome analysis of capacitated human sperm. Evidence of tyrosine phosphorylation of a kinase-anchoring protein 3 and valosin-containing protein/p97 during capacitation. *J Biol Chem* 278:11579-11589.
- Ficarro SB, McClelland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM (2002). Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat Biotechnol* 20:301-305.
- Fireman E, Shahar I, Shoval S, Messer G, Dvash S, Grief J (2001). Morphological and biochemical properties of alveolar fibroblasts in interstitial lung diseases. *Lung* 179:105-117.
- Fleming IN, Elliott CM, Exton JH (1996). Differential translocation of rho family GTPases by lysophosphatidic acid, endothelin-1, and platelet-derived growth factor. *J Biol Chem* 271:33067-33073.

- Force T, Kyriakis JM, Avruch J, Bonventre JV (1991). Endothelin, vasopressin, and angiotensin II enhance tyrosine phosphorylation by protein kinase C-dependent and -independent pathways in glomerular mesangial cells. *J Biol Chem* 266:6650-6656.
- Foster LJ, De Hoog CL, Mann M (2003). Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proc Natl Acad Sci U S A* 100:5813-5818.
- Freedman NJ, Ament AS, Oppermann M, Stoffel RH, Exum ST, Lefkowitz RJ (1997). Phosphorylation and desensitization of human endothelin A and B receptors. Evidence for G protein-coupled receptor kinase specificity. *J Biol Chem* 272:17734-17743.
- Freiden PJ, Gaut JR, Hendershot LM (1992). Interconversion of three differentially modified and assembled forms of BiP. *EMBO J* 11:63-70.
- Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY (1996). Control of adhesion-dependent cell survival by focal adhesion kinase. *J Cell Biol* 134:793-799.
- Fujihara H, Walker LA, Gong MC, Lemichez E, Boquet P, Somlyo AV, Somlyo AP (1997). Inhibition of RhoA translocation and calcium sensitization by in vivo ADP-ribosylation with the chimeric toxin DC3B. *Mol Biol Cell* 8:2437-2447.
- Furuhashi K (2002). Involvement of actin dephosphorylation in germination of *Physarum sclerotium*. *J Eukaryot Microbiol* 49:129-133.
- Gachet Y, Tournier S, Lee M, Lazaris-Karatzas A, Poulton T, Bommer UA (1999). The growth-related, translationally controlled protein P23 has properties of a tubulin binding protein and associates transiently with microtubules during the cell cycle. *J Cell Sci* 112 (Pt 8):1257-1271.
- Gauthier ML, Lydan MA, O'Day D, Cotter AD (1997). Endogenous autoinhibitors regulate changes in actin tyrosine phosphorylation during *Dictyostelium* spore germination. *Cell Signal* 9:79-83.

Ge Y, Rajkumar L, Guzman RC, Nandi S, Patton WF, Agnew BJ (2004). Multiplexed fluorescence detection of phosphorylation, glycosylation, and total protein in the proteomic analysis of breast cancer refractoriness. *Proteomics* 4:3464-3467.

Gellai M, Fletcher T, Pullen M, Nambi P (1996). Evidence for the existence of endothelin-B receptor subtypes and their physiological roles in the rat. *Am J Physiol* 271:R254-R261.

Gillespie MN, Owasoyo JO, McMurtry IF, O'Brien RF (1986). Sustained coronary vasoconstriction provoked by a peptidergic substance released from endothelial cells in culture. *J Pharmacol Exp Ther* 236:339-343.

Godovac-Zimmermann J, Soskic V, Poznanovic S, Brianza F (1999). Functional proteomics of signal transduction by membrane receptors. *Electrophoresis* 20:952-961.

Godovac-Zimmermann J, Brown LR (2001). Perspectives for mass spectrometry and functional proteomics. *Mass Spectrom Rev* 20:1-57.

Godovac-Zimmermann J, Brown LR (2003). Proteomics approaches to elucidation of signal transduction pathways. *Curr Opin Mol Ther* 5:241-249.

Gohla A, Offermanns S, Wilkie TM, Schultz G (1999). Differential involvement of G α 12 and G α 13 in receptor-mediated stress fiber formation. *J Biol Chem* 274:17901-17907.

Goldenring JR, Casanova JE, DeLorenzo RJ (1984). Tubulin-associated calmodulin-dependent kinase: evidence for an endogenous complex of tubulin with a calcium-calmodulin-dependent kinase. *J Neurochem* 43:1669-1679.

Goldie RG (1999). Endothelins in health and disease: an overview. *Clin Exp Pharmacol Physiol* 26:145-148.

- Goligorsky MS, Budzikowski AS, Tsukahara H, Noiri E (1999). Co-operation between endothelin and nitric oxide in promoting endothelial cell migration and angiogenesis. *Clin Exp Pharmacol Physiol* 26:269-271.
- Goodman OB, Jr., Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, Benovic JL (1996). Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature* 383:447-450.
- Goodnight JA, Mischak H, Kolch W, Mushinski JF (1995). Immunocytochemical localization of eight protein kinase C isozymes overexpressed in NIH 3T3 fibroblasts. Isoform-specific association with microfilaments, Golgi, endoplasmic reticulum, and nuclear and cell membranes. *J Biol Chem* 270:9991-10001.
- Gorvel JP, Chang TC, Boretto J, Azuma T, Chavrier P (1998). Differential properties of D4/LyGDI versus RhoGDI: phosphorylation and rho GTPase selectivity. *FEBS Lett* 422:269-273.
- Goto K, Kasuya Y, Matsuki N, Takuwa Y, Kurihara H, Ishikawa T, Kimura S, Yanagisawa M, Masaki T (1989). Endothelin activates the dihydropyridine-sensitive, voltage-dependent Ca²⁺ channel in vascular smooth muscle. *Proc Natl Acad Sci U S A* 86:3915-3918.
- Gregan B, Schaefer M, Rosenthal W, Oksche A (2004 a). Fluorescence Resonance Energy Transfer Analysis Reveals the Existence of Endothelin-A and Endothelin-B Receptor Homodimers. *J Cardiovasc Pharmacol* 44:S30-S33.
- Gregan B, Jurgensen J, Papsdorf G, Furkert J, Schaefer M, Beyermann M, Rosenthal W, Oksche A (2004 b). Ligand-dependent differences in the internalization of endothelin A and endothelin B receptor heterodimers. *J Biol Chem* 279:27679-27687.
- Griffin TJ, Gygi SP, Ideker T, Rist B, Eng J, Hood L, Aebersold R (2002). Complementary profiling of gene expression at the transcriptome and proteome levels in *Saccharomyces cerevisiae*. *Mol Cell Proteomics* 1:323-333.

- Grizot S, Faure J, Fieschi F, Vignais PV, Dagher MC, Pebay-Peyroula E (2001). Crystal structure of the Rac1-RhoGDI complex involved in nadph oxidase activation. *Biochemistry* 40:10007-10013.
- Gu L, Zhang H, Chen Q, Chen J (2003). Calyculin A-induced actin phosphorylation and depolymerization in renal epithelial cells. *Cell Motil Cytoskeleton* 54:286-295.
- Gudermann T, Grosse R, Schultz G (2000). Contribution of receptor/G protein signaling to cell growth and transformation. *Naunyn Schmiedebergs Arch Pharmacol* 361:345-362.
- Guerini D (1998). The significance of the isoforms of plasma membrane calcium ATPase. *Cell Tissue Res* 292:191-197.
- Guo GG, Patel K, Kumar V, Shah M, Fried VA, Etlinger JD, Sehgal PB (2002). Association of the chaperone glucose-regulated protein 58 (GRP58/ER-60/ERp57) with Stat3 in cytosol and plasma membrane complexes. *J Interferon Cytokine Res* 22:555-563.
- Guo W, Shi L, Javitch JA (2003). The fourth transmembrane segment forms the interface of the dopamine D2 receptor homodimer. *J Biol Chem* 278:4385-4388.
- Gutkind JS (1998). The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. *J Biol Chem* 273:1839-1842.
- Gygi SP, Rochon Y, Franza BR, Aebersold R (1999). Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* 19:1720-1730.
- Haendler B, Hechler U, Schleuning WD (1992). Molecular cloning of human endothelin (ET) receptors ETA and ETB. *J Cardiovasc Pharmacol* 20 Suppl 12:S1-S4.

- Hafizi S, Allen SP, Goodwin AT, Chester AH, Yacoub MH (1999). Endothelin-1 stimulates proliferation of human coronary smooth muscle cells via the ET(A) receptor and is co-mitogenic with growth factors. *Atherosclerosis* 146:351-359.
- Hahn AW, Regenass S, Resink TJ, Kern F, Buhler FR (1993). Morphogenic effects of endothelin-1 on vascular smooth muscle cells. *J Vasc Res* 30:192-201.
- Hall RA, Premont RT, Lefkowitz RJ (1999). Heptahelical receptor signaling: beyond the G protein paradigm. *J Cell Biol* 145:927-932.
- Hamm HE (1998). The many faces of G protein signaling. *J Biol Chem* 273:669-672.
- Hannan KM, Hannan RD, Rothblum LI (1998). Transcription by RNA polymerase I. *Front Biosci* 3:d376-d398.
- Hannan KM, Hannan RD, Smith SD, Jefferson LS, Lun M, Rothblum LI (2000). Rb and p130 regulate RNA polymerase I transcription: Rb disrupts the interaction between UBF and SL-1. *Oncogene* 19:4988-4999.
- Harding MW, Galat A, Uehling DE, Schreiber SL (1989). A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. *Nature* 341:758-760.
- Hawkins TE, Merrifield CJ, Moss SE (2000). Calcium signaling and annexins. *Cell Biochem Biophys* 33:275-296.
- Hay DW (1999). Putative mediator role of endothelin-1 in asthma and other lung diseases. *Clin Exp Pharmacol Physiol* 26:168-171.
- Hellman U, Wernstedt C, Gonez J, Heldin CH (1995). Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. *Anal Biochem* 224:451-455.

- Hendershot LM, Ting J, Lee AS (1988). Identity of the immunoglobulin heavy-chain-binding protein with the 78,000-dalton glucose-regulated protein and the role of posttranslational modifications in its binding function. *Mol Cell Biol* 8:4250-4256.
- Her JH, Lakhani S, Zu K, Vila J, Dent P, Sturgill TW, Weber MJ (1993). Dual phosphorylation and autophosphorylation in mitogen-activated protein (MAP) kinase activation. *Biochem J* 296 (Pt 1):25-31.
- Herman WH, Simonson MS (1995). Nuclear signaling by endothelin-1. A Ras pathway for activation of the c-fos serum response element. *J Biol Chem* 270:11654-11661.
- Herrera M, Garvin JL (2004). Endothelin stimulates endothelial nitric oxide synthase expression in the thick ascending limb. *Am J Physiol Renal Physiol* 287:F231-F235.
- Hickey KA, Rubanyi G, Paul RJ, Highsmith RF (1985). Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. *Am J Physiol* 248:C550-C556.
- Higuchi T, Watanabe Y, Waga I (2004). Protein disulfide isomerase suppresses the transcriptional activity of NF-kappaB. *Biochem Biophys Res Commun* 318:46-52.
- Hilal-Dandan R, Ramirez MT, Villegas S, Gonzalez A, Endo-Mochizuki Y, Brown JH, Brunton LL (1997). Endothelin ETA receptor regulates signaling and ANF gene expression via multiple G protein-linked pathways. *Am J Physiol* 272:H130-H137.
- Hingorani K, Szebeni A, Olson MO (2000). Mapping the functional domains of nucleolar protein B23. *J Biol Chem* 275:24451-24457.
- Hirao M, Sato N, Kondo T, Yonemura S, Monden M, Sasaki T, Takai Y, Tsukita S, Tsukita S (1996). Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway. *J Cell Biol* 135:37-51.

Hirshman CA, Emala CW (1999). Actin reorganization in airway smooth muscle cells involves Gq and Gi-2 activation of Rho. *Am J Physiol* 277:L653-L661.

Hoche B, Thone-Reineke C, Bauer C, Raschack M, Neumayer HH (1997). The paracrine endothelin system: pathophysiology and implications in clinical medicine. *Eur J Clin Chem Clin Biochem* 35:175-189.

Hosoda K, Hammer RE, Richardson JA, Baynash AG, Cheung JC, Giaid A, Yanagisawa M (1994). Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. *Cell* 79:1267-1276.

Hua H, Munk S, Whiteside CI (2003). Endothelin-1 activates mesangial cell ERK1/2 via EGF-receptor transactivation and caveolin-1 interaction. *Am J Physiol Renal Physiol* 284:F303-F312.

Hughes TR, Shoemaker DD (2001). DNA microarrays for expression profiling. *Curr Opin Chem Biol* 5:21-25.

Hunter T (1995). Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* 80:225-236.

Hunter T (1998). The Croonian Lecture 1997. The phosphorylation of proteins on tyrosine: its role in cell growth and disease. *Philos Trans R Soc Lond B Biol Sci* 353:583-605.

Hur EM, Kim KT (2002). G protein-coupled receptor signalling and cross-talk: achieving rapidity and specificity. *Cell Signal* 14:397-405.

Hyvelin JM, Guibert C, Marthan R, Savineau JP (1998). Cellular mechanisms and role of endothelin-1-induced calcium oscillations in pulmonary arterial myocytes. *Am J Physiol* 275:L269-L282.

- Ibarrola N, Molina H, Iwahori A, Pandey A (2004). A novel proteomic approach for specific identification of tyrosine kinase substrates using [¹³C]tyrosine. *J Biol Chem* 279:15805-15813.
- Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* 377:539-544.
- Imamura T, Huang J, Dalle S, Ugi S, Usui I, Luttrell LM, Miller WE, Lefkowitz RJ, Olefsky JM (2001). beta -Arrestin-mediated recruitment of the Src family kinase Yes mediates endothelin-1-stimulated glucose transport. *J Biol Chem* 276:43663-43667.
- Immler D, Gremm D, Kirsch D, Spengler B, Presek P, Meyer HE (1998). Identification of phosphorylated proteins from thrombin-activated human platelets isolated by two-dimensional gel electrophoresis by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) and liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). *Electrophoresis* 19:1015-1023.
- Inada H, Togashi H, Nakamura Y, Kaibuchi K, Nagata K, Inagaki M (1999). Balance between activities of Rho kinase and type 1 protein phosphatase modulates turnover of phosphorylation and dynamics of desmin/vimentin filaments. *J Biol Chem* 274:34932-34939.
- Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, Masaki T (1989). The human endothelin family: three structurally and pharmacologically distinct isoptides predicted by three separate genes. *Proc Natl Acad Sci U S A* 86:2863-2867.
- Ishikawa K, Ihara M, Noguchi K, Mase T, Mino N, Saeki T, Fukuroda T, Fukami T, Ozaki S, Nagase T, . (1994). Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. *Proc Natl Acad Sci U S A* 91:4892-4896.

- Itoh S, Sasaki T, Ide K, Ishikawa K, Nishikibe M, Yano M (1993). A novel endothelin ETA receptor antagonist, BQ-485, and its preventive effect on experimental cerebral vasospasm in dogs. *Biochem Biophys Res Commun* 195:969-975.
- Iwamuro Y, Miwa S, Zhang XF, Minowa T, Enoki T, Okamoto Y, Hasegawa H, Furutani H, Okazawa M, Ishikawa M, Hashimoto N, Masaki T (1999). Activation of three types of voltage-independent Ca²⁺ channel in A7r5 cells by endothelin-1 as revealed by a novel Ca²⁺ channel blocker LOE 908. *Br J Pharmacol* 126:1107-1114.
- Iwasaki H, Eguchi S, Ueno H, Marumo F, Hirata Y (1999). Endothelin-mediated vascular growth requires p42/p44 mitogen-activated protein kinase and p70 S6 kinase cascades via transactivation of epidermal growth factor receptor. *Endocrinology* 140:4659-4668.
- Jaattela M (1999). Escaping cell death: survival proteins in cancer. *Exp Cell Res* 248:30-43.
- James PH, Pruschy M, Vorherr TE, Penniston JT, Carafoli E (1989). Primary structure of the cAMP-dependent phosphorylation site of the plasma membrane calcium pump. *Biochemistry* 28:4253-4258.
- Jin WH, Dai J, Zhou H, Xia QC, Zou HF, Zeng R (2004). Phosphoproteome analysis of mouse liver using immobilized metal affinity purification and linear ion trap mass spectrometry. *Rapid Commun Mass Spectrom* 18:2169-2176.
- Johnson GL, Lapadat R (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298:1911-1912.
- Josko J, Hendryk S, Jedrzejowska-Szypulka H, Slowinski J, Gwozdz B, Lange D, Snietura M, Zwirska-Korczala K, Jochem J (2001). Cerebral angiogenesis after subarachnoid hemorrhage (SAH) and endothelin receptor blockage with BQ-123 antagonist in rats. *J Physiol Pharmacol* 52:237-248.

- Jouneaux C, Goldsmith P, Hanoune J, Lotersztajn S (1993). Endothelin inhibits the calcium pump and stimulates phosphoinositide phospholipase C in liver plasma membranes via two different G proteins, Gs and Gq. *J Cardiovasc Pharmacol* 22 Suppl 8:S158-S160.
- Jouneaux C, Mallat A, Serradeil-Le Gal C, Goldsmith P, Hanoune J, Lotersztajn S (1994). Coupling of endothelin B receptors to the calcium pump and phospholipase C via Gs and Gq in rat liver. *J Biol Chem* 269:1845-1851.
- Just A, Olson AJ, Arendshorst WJ (2004). Dual constrictor and dilator actions of ET(B) receptors in the rat renal microcirculation: interactions with ET(A) receptors. *Am J Physiol Renal Physiol* 286:F660-F668.
- Kai H, Kanaide H, Nakamura M (1989). Endothelin-sensitive intracellular Ca²⁺ store overlaps with caffeine-sensitive one in rat aortic smooth muscle cells in primary cultures. *Biochem Biophys Res Commun* 158:235-243.
- Kamijo R, Harada H, Matsuyama T, Bosland M, Gerecitano J, Shapiro D, Le J, Koh SI, Kimura T, Green SJ, . (1994). Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science* 263:1612-1615.
- Kanamoto T, Hellman U, Heldin CH, Souchelnytskyi S (2002). Functional proteomics of transforming growth factor-beta1-stimulated Mv1Lu epithelial cells: Rad51 as a target of TGFbeta1-dependent regulation of DNA repair. *EMBO J* 21:1219-1230.
- Kanashiro CA, Altirkawi KA, Khalil RA (2000). Preconditioning of coronary artery against vasoconstriction by endothelin-1 and prostaglandin F2alpha during repeated down-regulation of epsilon-protein kinase C. *J Cardiovasc Pharmacol* 35:491-501.
- Kang S, Liao P, Gage DA, Esselman WJ (1997). Identification of in vivo phosphorylation sites of CD45 protein-tyrosine phosphatase in 70Z/3.12 cells. *J Biol Chem* 272:11588-11596.

- Kapeller R, Chakrabarti R, Cantley L, Fay F, Corvera S (1993). Internalization of activated platelet-derived growth factor receptor-phosphatidylinositol-3' kinase complexes: potential interactions with the microtubule cytoskeleton. *Mol Cell Biol* 13:6052-6063.
- Karne S, Jayawickreme CK, Lerner MR (1993). Cloning and characterization of an endothelin-3 specific receptor (ETC receptor) from *Xenopus laevis* dermal melanophores. *J Biol Chem* 268:19126-19133.
- Katahira R, Flotow H, Thomas G, Nosaka AY (1996). Solution structure of the phosphorylated sites of ribosomal protein S6 by ¹H NMR spectroscopy. *Int J Pept Protein Res* 47:282-288.
- Kato K, Asai R, Shimizu A, Suzuki F, Ariyoshi Y (1983). Immunoassay of three enolase isozymes in human serum and in blood cells. *Clin Chim Acta* 127:353-363.
- Kautz B, Kakar R, David E, Eklund EA (2001). SHP1 protein-tyrosine phosphatase inhibits gp91PHOX and p67PHOX expression by inhibiting interaction of PU.1, IRF1, interferon consensus sequence-binding protein, and CREB-binding protein with homologous Cis elements in the CYBB and NCF2 genes. *J Biol Chem* 276:37868-37878.
- Kawanabe Y, Hashimoto N, Masaki T (2002). Effects of phosphoinositide 3-kinase on the endothelin-1-induced activation of voltage-independent Ca(2+) channels and mitogenesis in Chinese hamster ovary cells stably expressing endothelin(a) receptor. *Mol Pharmacol* 62:756-761.
- Kawanabe Y, Hashimoto N, Masaki T (2003). Involvements of voltage-independent Ca²⁺ channels and phosphoinositide 3-kinase in endothelin-1-induced PYK2 tyrosine phosphorylation. *Mol Pharmacol* 63:808-813.

Kawanabe Y, Hashimoto N, Masaki T (2004). Effects of phosphoinositide 3-kinase on endothelin-1-induced activation of voltage-independent Ca^{2+} channels and vasoconstriction. *Biochem Pharmacol* 68:215-221.

Keeton TP, Burk SE, Shull GE (1993). Alternative splicing of exons encoding the calmodulin-binding domains and C termini of plasma membrane Ca^{2+} -ATPase isoforms 1, 2, 3, and 4. *J Biol Chem* 268:2740-2748.

Kim JH, Cho YS, Kim BC, Kim YS, Lee GS (1997). Role of Rho GTPase in the endothelin-1-induced nuclear signaling. *Biochem Biophys Res Commun* 232:223-226.

Kim M, Jung Y, Lee K, Kim C (2000). Identification of the calcium binding sites in translationally controlled tumor protein. *Arch Pharm Res* 23:633-636.

Klein J, Grummt I (1999). Cell cycle-dependent regulation of RNA polymerase I transcription: the nucleolar transcription factor UBF is inactive in mitosis and early G1. *Proc Natl Acad Sci U S A* 96:6096-6101.

Kohan DE (1997). Endothelins in the normal and diseased kidney. *Am J Kidney Dis* 29:2-26.

Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, Rapp UR (1993). Protein kinase C α activates RAF-1 by direct phosphorylation. *Nature* 364:249-252.

Kolker E, Picone AF, Galperin MY, Romine MF, Higdon R, Makarova KS, Kolker N, Anderson GA, Qiu X, Auberry KJ, Babnigg G, Beliaev AS, Edlefsen P, Elias DA, Gorby YA, Holzman T, Klappenbach JA, Konstantinidis KT, Land ML, Lipton MS, McCue LA, Monroe M, Pasa-Tolic L, Pinchuk G, Purvine S, Serres MH, Tsapin S, Zakrajsek BA, Zhu W, Zhou J, Larimer FW, Lawrence CE, Riley M, Collart FR, Yates JR, III, Smith RD, Giometti CS, Nealson KH, Fredrickson JK, Tiedje JM (2005). Global profiling of *Shewanella oneidensis* MR-1: expression of hypothetical genes and improved functional annotations. *Proc Natl Acad Sci U S A* 102:2099-2104.

Koyama Y, Baba A (1999). Endothelin-induced protein tyrosine phosphorylation of cultured astrocytes: its relationship to cytoskeletal actin organization. *Glia* 26:324-332.

Krebs EG (1994). The growth of research on protein phosphorylation. *Trends Biochem Sci* 19:439.

Krijgsveld J, Ketting RF, Mahmoudi T, Johansen J, Artal-Sanz M, Verrijzer CP, Plasterk RH, Heck AJ (2003). Metabolic labeling of *C. elegans* and *D. melanogaster* for quantitative proteomics. *Nat Biotechnol* 21:927-931.

Kroger A, Koster M, Schroeder K, Hauser H, Mueller PP (2002). Activities of IRF-1. *J Interferon Cytokine Res* 22:5-14.

Kurihara Y, Kurihara H, Suzuki H, Kodama T, Maemura K, Nagai R, Oda H, Kuwaki T, Cao WH, Kamada N, . (1994). Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. *Nature* 368:703-710.

Kurihara Y, Kurihara H, Maemura K, Kuwaki T, Kumada M, Yazaki Y (1995). Impaired development of the thyroid and thymus in endothelin-1 knockout mice. *J Cardiovasc Pharmacol* 26 Suppl 3:S13-S16.

Kusuhara M, Yamaguchi K, Kuranami M, Suzaki A, Ishikawa S, Moon H, Adachi I, Hori S, Handa S (1992). Stimulation of anchorage-independent cell growth by endothelin in NRK 49F cells. *Cancer Res* 52:3011-3014.

- Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Lahav R, Ziller C, Dupin E, Le Douarin NM (1996). Endothelin 3 promotes neural crest cell proliferation and mediates a vast increase in melanocyte number in culture. *Proc Natl Acad Sci U S A* 93:3892-3897.
- Lahaye DH, Walboomers F, Peters PH, Theuvenet AP, Van Zoelen EJ (1999). Phenotypic transformation of normal rat kidney fibroblasts by endothelin-1. Different mode of action from lysophosphatidic acid, bradykinin, and prostaglandin f2alpha. *Biochim Biophys Acta* 1449:107-118.
- Leader DP (1980). Phosphorylated and other modified forms of eukaryotic ribosomal protein S3 analysed by two-dimensional gel electrophoresis. *Biochem J* 189:241-245.
- Leopoldt D, Yee HF, Jr., Saab S, Rozengurt E (2000). Tyrosine phosphorylation of p125(Fak), p130(Cas), and paxillin does not require extracellular signal-regulated kinase activation in Swiss 3T3 cells stimulated by bombesin or platelet-derived growth factor. *J Cell Physiol* 183:208-220.
- Leustek T, Toledo H, Brot N, Weissbach H (1991). Calcium-dependent autophosphorylation of the glucose-regulated protein, Grp78. *Arch Biochem Biophys* 289:256-261.
- Li Y, Camacho P (2004). Ca²⁺-dependent redox modulation of SERCA 2b by ERp57. *J Cell Biol* 164:35-46.
- Liao PC, Leykam J, Andrews PC, Gage DA, Allison J (1994). An approach to locate phosphorylation sites in a phosphoprotein: mass mapping by combining specific enzymatic degradation with matrix-assisted laser desorption/ionization mass spectrometry. *Anal Biochem* 219:9-20.

- Lin R, Hiscott J (1999). A role for casein kinase II phosphorylation in the regulation of IRF-1 transcriptional activity. *Mol Cell Biochem* 191:169-180.
- Lindquist JA, Hammerling GJ, Trowsdale J (2001). ER60/ERp57 forms disulfide-bonded intermediates with MHC class I heavy chain. *FASEB J* 15:1448-1450.
- Liu H, Stupak J, Zheng J, Keller BO, Brix BJ, Fliegel L, Li L (2004). Open tubular immobilized metal ion affinity chromatography combined with MALDI MS and MS/MS for identification of protein phosphorylation sites. *Anal Chem* 76:4223-4232.
- Liu Y, Geisbuhler B, Jones AW (1992). Activation of multiple mechanisms including phospholipase D by endothelin-1 in rat aorta. *Am J Physiol* 262:C941-C949.
- Luciano LG, D'Orleans-Juste P, Calixto JB, Rae GA (1998). Endothelin-1 selectively potentiates the purinergic component of sympathetic neurotransmission in rat seminal vesicle. *J Cardiovasc Pharmacol* 31 Suppl 1:S515-S517.
- Lunn JA, Wong H, Rozengurt E, Walsh JH (2000). Requirement of cortical actin organization for bombesin, endothelin, and EGF receptor internalization. *Am J Physiol Cell Physiol* 279:C2019-C2027.
- Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ (1999). Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* 283:655-661.
- MacCumber MW, Ross CA, Glaser BM, Snyder SH (1989). Endothelin: visualization of mRNAs by in situ hybridization provides evidence for local action. *Proc Natl Acad Sci U S A* 86:7285-7289.
- Magdalena J, Millard TH, Machesky LM (2003). Microtubule involvement in NIH 3T3 Golgi and MTOC polarity establishment. *J Cell Sci* 116:743-756.

- Marrero MB, Schieffer B, Paxton WG, Heerdt L, Berk BC, Delafontaine P, Bernstein KE (1995). Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. *Nature* 375:247-250.
- Matsumoto H, Uemasu J, Kitano M, Kawasaki H (1994). Clinical significance of plasma endothelin-1 in patients with chronic liver disease. *Dig Dis Sci* 39:2665-2670.
- Matten WT, Aubry M, West J, Maness PF (1990). Tubulin is phosphorylated at tyrosine by pp60c-src in nerve growth cone membranes. *J Cell Biol* 111:1959-1970.
- Megidish T, Takio K, Titani K, Iwabuchi K, Hamaguchi A, Igarashi Y, Hakomori S (1999). Endogenous substrates of sphingosine-dependent kinases (SDKs) are chaperone proteins: heat shock proteins, glucose-regulated proteins, protein disulfide isomerase, and calreticulin. *Biochemistry* 38:3369-3378.
- Mehta D, Rahman A, Malik AB (2001). Protein kinase C- α signals rho-guanine nucleotide dissociation inhibitor phosphorylation and rho activation and regulates the endothelial cell barrier function. *J Biol Chem* 276:22614-22620.
- Menard S, Tagliabue E, Colnaghi MI (1998). The 67 kDa laminin receptor as a prognostic factor in human cancer. *Breast Cancer Res Treat* 52:137-145.
- Miklos GL, Maleszka R (2001). Integrating molecular medicine with functional proteomics: realities and expectations. *Proteomics* 1:30-41.
- Minta A, Kao JP, Tsien RY (1989). Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J Biol Chem* 264:8171-8178.
- Miyamoto Y, Yamauchi J, Itoh H (2003). Src kinase regulates the activation of a novel FGD-1-related Cdc42 guanine nucleotide exchange factor in the signaling pathway from the endothelin A receptor to JNK. *J Biol Chem* 278:29890-29900.

- Momose H, Kurosu H, Tsujimoto N, Kontani K, Tsujita K, Nishina H, Katada T (2003). Dual phosphorylation of phosphoinositide 3-kinase adaptor Grb2-associated binder 2 is responsible for superoxide formation synergistically stimulated by Fc gamma and formyl-methionyl-leucyl-phenylalanine receptors in differentiated THP-1 cells. *J Immunol* 171:4227-4234.
- Naidoo V, Naidoo S, Raidoo DM (2004). Immunolocalisation of endothelin-1 in human brain. *J Chem Neuroanat* 27:193-200.
- Neer EJ (1995). Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* 80:249-257.
- Nelson J, Bagnato A, Battistini B, Nisen P (2003). The endothelin axis: emerging role in cancer. *Nat Rev Cancer* 3:110-116.
- Nelson JB, Hedican SP, George DJ, Reddi AH, Piantadosi S, Eisenberger MA, Simons JW (1995). Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. *Nat Med* 1:944-949.
- Neville DC, Rozanas CR, Price EM, Gruis DB, Verkman AS, Townsend RR (1997). Evidence for phosphorylation of serine 753 in CFTR using a novel metal-ion affinity resin and matrix-assisted laser desorption mass spectrometry. *Protein Sci* 6:2436-2445.
- Neyses L, Nouskas J, Vetter H (1991). Inhibition of endothelin-1 induced myocardial protein synthesis by an antisense oligonucleotide against the early growth response gene-1. *Biochem Biophys Res Commun* 181:22-27.
- Nishikawa K, Toker A, Johannes FJ, Songyang Z, Cantley LC (1997). Determination of the specific substrate sequence motifs of protein kinase C isozymes. *J Biol Chem* 272:952-960.
- Noiva R (1999). Protein disulfide isomerase: the multifunctional redox chaperone of the endoplasmic reticulum. *Semin Cell Dev Biol* 10:481-493.

- Nuhse TS, Stensballe A, Jensen ON, Peck SC (2003). Large-scale Analysis of in Vivo Phosphorylated Membrane Proteins by Immobilized Metal Ion Affinity Chromatography and Mass Spectrometry. *Mol Cell Proteomics* 2:1234-1243.
- O'Brien RF, Robbins RJ, McMurtry IF (1987). Endothelial cells in culture produce a vasoconstrictor substance. *J Cell Physiol* 132:263-270.
- O'Farrell PH (1975). High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250:4007-4021.
- Obara K, Hata S, Sato K, Koide M, Ishii K, Nakayama K (1999). Contractile potentiation by endothelin-1 involves protein kinase C-delta activity in the porcine coronary artery. *Jpn J Physiol* 49:175-183.
- Ohno S, Kawasaki H, Imajoh S, Suzuki K, Inagaki M, Yokokura H, Sakoh T, Hidaka H (1987). Tissue-specific expression of three distinct types of rabbit protein kinase C. *Nature* 325:161-166.
- Okamoto Y, Ninomiya H, Miwa S, Masaki T (2000). Cholesterol oxidation switches the internalization pathway of endothelin receptor type A from caveolae to clathrin-coated pits in Chinese hamster ovary cells. *J Biol Chem* 275:6439-6446.
- Okazawa M, Shiraki T, Ninomiya H, Kobayashi S, Masaki T (1998). Endothelin-induced apoptosis of A375 human melanoma cells. *J Biol Chem* 273:12584-12592.
- Okuda M, Horn HF, Tarapore P, Tokuyama Y, Smulian AG, Chan PK, Knudsen ES, Hofmann IA, Snyder JD, Bove KE, Fukasawa K (2000). Nucleophosmin/B23 is a target of CDK2/cyclin E in centrosome duplication. *Cell* 103:127-140.
- Olofsson B (1999). Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling. *Cell Signal* 11:545-554.

Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M (2002). Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics* 1:376-386.

Ono K, Han J (2000). The p38 signal transduction pathway: activation and function. *Cell Signal* 12:1-13.

Otsu M, Urade R, Kito M, Omura F, Kikuchi M (1995). A possible role of ER-60 protease in the degradation of misfolded proteins in the endoplasmic reticulum. *J Biol Chem* 270:14958-14961.

Otto H, Dreger M, Bengtsson L, Hucho F (2001). Identification of tyrosine-phosphorylated proteins associated with the nuclear envelope. *Eur J Biochem* 268:420-428.

Pagano A, Rovelli G, Mosbacher J, Lohmann T, Duthey B, Stauffer D, Ristig D, Schuler V, Meigel I, Lampert C, Stein T, Prezeau L, Blahos J, Pin J, Froestl W, Kuhn R, Heid J, Kaupmann K, Bettler B (2001). C-terminal interaction is essential for surface trafficking but not for heteromeric assembly of GABA(b) receptors. *J Neurosci* 21:1189-1202.

Pandey A, Blagoev B, Kratchmarova I, Fernandez M, Nielsen M, Kristiansen TZ, Ohara O, Podtelejnikov AV, Roche S, Lodish HF, Mann M (2002). Cloning of a novel phosphotyrosine binding domain containing molecule, Odin, involved in signaling by receptor tyrosine kinases. *Oncogene* 21:8029-8036.

Pasa-Tolic L, Lipton MS, Masselon CD, Anderson GA, Shen Y, Tolic N, Smith RD (2002). Gene expression profiling using advanced mass spectrometric approaches. *J Mass Spectrom* 37:1185-1198.

Patterson CE, Schaub T, Coleman EJ, Davis EC (2000). Developmental regulation of FKBP65. An ER-localized extracellular matrix binding-protein. *Mol Biol Cell* 11:3925-3935.

- Patton WF (2002). Detection technologies in proteome analysis. *J Chromatogr B Analyt Technol Biomed Life Sci* 771:3-31.
- Pawson T, Nash P (2003). Assembly of cell regulatory systems through protein interaction domains. *Science* 300:445-452.
- Peake P, Winter N, Britton W (1998). Phosphorylation of Mycobacterium leprae heat-shock 70 protein at threonine 175 alters its substrate binding characteristics. *Biochim Biophys Acta* 1387:387-394.
- Pedram A, Razandi M, Hu RM, Levin ER (1998). Astrocyte progression from G1 to S phase of the cell cycle depends upon multiple protein interaction. *J Biol Chem* 273:13966-13972.
- Peeler TC, Conrad KM, Baker KM (1996). Endothelin stimulates sis-inducing factor-like DNA binding activity in CHO-K1 cells expressing ETA receptors. *Biochem Biophys Res Commun* 221:62-66.
- Pelletier G, Stefanovsky VY, Faublader M, Hirschler-Laszkiewicz I, Savard J, Rothblum LI, Cote J, Moss T (2000). Competitive recruitment of CBP and Rb-HDAC regulates UBF acetylation and ribosomal transcription. *Mol Cell* 6:1059-1066.
- Petit V, Thiery JP (2000). Focal adhesions: structure and dynamics. *Biol Cell* 92:477-494.
- Pitcher JA, Hall RA, Daaka Y, Zhang J, Ferguson SS, Hester S, Miller S, Caron MG, Lefkowitz RJ, Barak LS (1998). The G protein-coupled receptor kinase 2 is a microtubule-associated protein kinase that phosphorylates tubulin. *J Biol Chem* 273:12316-12324.

- Polanowska-Grabowska R, Simon CG, Jr., Falchetto R, Shabanowitz J, Hunt DF, Gear AR (1997). Platelet adhesion to collagen under flow causes dissociation of a phosphoprotein complex of heat-shock proteins and protein phosphatase 1. *Blood* 90:1516-1526.
- Pollock DM, Keith TL, Highsmith RF (1995). Endothelin receptors and calcium signaling. *FASEB J* 9:1196-1204.
- Pollock DM (1998). Endothelin receptor subtypes and tissue distribution. *Endothelin* 1-29.
- Polte TR, Hanks SK (1997). Complexes of focal adhesion kinase (FAK) and Crk-associated substrate (p130(Cas)) are elevated in cytoskeleton-associated fractions following adhesion and Src transformation. Requirements for Src kinase activity and FAK proline-rich motifs. *J Biol Chem* 272:5501-5509.
- Polyak K, Lee MH, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P, Massague J (1994). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* 78:59-66.
- Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, Ullrich A (1999). EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* 402:884-888.
- Pribnow D, Muldoon LL, Fajardo M, Theodor L, Chen LY, Magun BE (1992). Endothelin induces transcription of fos/jun family genes: a prominent role for calcium ion. *Mol Endocrinol* 6:1003-1012.
- Quemeneur E, Guthapfel R, Gueguen P (1994). A major phosphoprotein of the endoplasmic reticulum is protein disulfide isomerase. *J Biol Chem* 269:5485-5488.
- Rabilloud T (2002). Two-dimensional gel electrophoresis in proteomics: old, old fashioned, but it still climbs up the mountains. *Proteomics* 2:3-10.

- Ramagli LS, Rodriguez LV (1985). Quantitation of Microgram Amounts of Protein in Two-Dimensional Polyacrylamide-Gel Electrophoresis Sample Buffer. *Electrophoresis* 6:559-563.
- Reiss K, Capasso JM, Huang HE, Meggs LG, Li P, Anversa P (1993). ANG II receptors, c-myc, and c-jun in myocytes after myocardial infarction and ventricular failure. *Am J Physiol* 264:H760-H769.
- Reiss N, Kanety H, Schlessinger J (1986). Five enzymes of the glycolytic pathway serve as substrates for purified epidermal-growth-factor-receptor kinase. *Biochem J* 239:691-697.
- Reiss N, Oplatka A, Hermon J, Naor Z (1996). Phosphatidylserine directs differential phosphorylation of actin and glyceraldehyde-3-phosphate dehydrogenase by protein kinase C: possible implications for regulation of actin polymerization. *Biochem Mol Biol Int* 40:1191-1200.
- Ridley AJ, Hall A (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 70:389-399.
- Robin P, Boulven I, Bole-Feysot C, Tanfin Z, Leiber D (2004). Contribution of PKC-dependent and -independent processes in temporal ERK regulation by ET-1, PDGF, and EGF in rat myometrial cells. *Am J Physiol Cell Physiol* 286:C798-C806.
- Rodland KD, Muldoon LL, Magun BE (1991). Regulation of intracellular Ca²⁺ and gene expression by endothelin-1. *J Cardiovasc Pharmacol* 17 Suppl 7:S89-S95.
- Roos M, Soskic V, Poznanovic S, Godovac-Zimmermann J (1998). Post-translational modifications of endothelin receptor B from bovine lungs analyzed by mass spectrometry. *J Biol Chem* 273:924-931.
- Roux S, Breu V, Ertel SI, Clozel M (1999). Endothelin antagonism with bosentan: a review of potential applications. *J Mol Med* 77:364-376.

Rubanyi GM, Polokoff MA (1994). Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol Rev* 46:325-415.

Rybin VO, Xu X, Steinberg SF (1999). Activated protein kinase C isoforms target to cardiomyocyte caveolae : stimulation of local protein phosphorylation. *Circ Res* 84:980-988.

Saida K, Mitsui Y (1991). Structure of the precursor for vasoactive intestinal contractor (VIC): its comparison with those of endothelin-1 and endothelin-3. *J Cardiovasc Pharmacol* 17 Suppl 7:S55-S58.

Sakai J, Ishikawa H, Kojima S, Satoh H, Yamamoto S, Kanaoka M (2003). Proteomic analysis of rat heart in ischemia and ischemia-reperfusion using fluorescence two-dimensional difference gel electrophoresis. *Proteomics* 3:1318-1324.

Sakai S, Miyauchi T, Kobayashi M, Yamaguchi I, Goto K, Sugishita Y (1996). Inhibition of myocardial endothelin pathway improves long-term survival in heart failure. *Nature* 384:353-355.

Sakimura K, Kushiya E, Takahashi Y, Suzuki Y (1987). The structure and expression of neuron-specific enolase gene. *Gene* 60:103-113.

Sakurada S, Okamoto H, Takuwa N, Sugimoto N, Takuwa Y (2001). Rho activation in excitatory agonist-stimulated vascular smooth muscle. *Am J Physiol Cell Physiol* 281:C571-C578.

Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, Masaki T (1990). Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* 348:732-735.

Sakurai T, Yanagisawa M, Masaki T (1992). Molecular characterization of endothelin receptors. *Trends Pharmacol Sci* 13:103-108.

- Sakurai T, Abe Y, Kasuya Y, Takuwa N, Shiba R, Yamashita T, Endo T, Goto K (1994). Activin A stimulates mitogenesis in Swiss 3T3 fibroblasts without activation of mitogen-activated protein kinases. *J Biol Chem* 269:14118-14122.
- Salani D, Di C, V, Nicotra MR, Rosano L, Tecce R, Venuti A, Natali PG, Bagnato A (2000). Role of endothelin-1 in neovascularization of ovarian carcinoma. *Am J Pathol* 157:1537-1547.
- Salazar EP, Rozengurt E (1999). Bombesin and platelet-derived growth factor induce association of endogenous focal adhesion kinase with Src in intact Swiss 3T3 cells. *J Biol Chem* 274:28371-28378.
- Sale EM, White MF, Kahn CR (1987). Phosphorylation of glycolytic and gluconeogenic enzymes by the insulin receptor kinase. *J Cell Biochem* 33:15-26.
- Sarman B, Toth M, Somogyi A (1998). Role of endothelin-1 in diabetes mellitus. *Diabetes Metab Rev* 14:171-175.
- Scherle P, Behrens T, Staudt LM (1993). Ly-GDI, a GDP-dissociation inhibitor of the RhoA GTP-binding protein, is expressed preferentially in lymphocytes. *Proc Natl Acad Sci U S A* 90:7568-7572.
- Schinelli S, Zanassi P, Paolillo M, Wang H, Feliciello A, Gallo V (2001). Stimulation of endothelin B receptors in astrocytes induces cAMP response element-binding protein phosphorylation and c-fos expression via multiple mitogen-activated protein kinase signaling pathways. *J Neurosci* 21:8842-8853.
- Schlaepfer DD, Hauck CR, Sieg DJ (1999). Signaling through focal adhesion kinase. *Prog Biophys Mol Biol* 71:435-478.

- Schramek H, Wang Y, Konieczkowski M, Rose PM, Sedor JR, Dunn MJ (1994). Endothelin-1 stimulates cytosolic phospholipase A2 in Chinese hamster ovary cells stably expressing the human ETA or ETB receptor subtype. *Biochem Biophys Res Commun* 199:992-997.
- Schulenberg B, Goodman TN, Aggeler R, Capaldi RA, Patton WF (2004). Characterization of dynamic and steady-state protein phosphorylation using a fluorescent phosphoprotein gel stain and mass spectrometry. *Electrophoresis* 25:2526-2532.
- Scott L, Lamb J, Smith S, Wheatley DN (2000). Single amino acid (arginine) deprivation: rapid and selective death of cultured transformed and malignant cells. *Br J Cancer* 83:800-810.
- Sernerer GG, Modesti PA, Cecioni I, Biagini D, Migliorini A, Costoli A, Colella A, Naldoni A, Paoletti P (1995). Plasma endothelin and renal endothelin are two distinct systems involved in volume homeostasis. *Am J Physiol* 268:H1829-H1837.
- Sernerer GG, Cecioni I, Vanni S, Panaccia R, Bandinelli B, Vetere A, Janming X, Bertolozzi I, Boddi M, Lisi GF, Sani G, Modesti PA (2000). Selective up-regulation of cardiac endothelin system in patients with ischemic but not idiopathic dilated cardiomyopathy: endothelin-1 system in the human failing heart. *Circ Res* 86:377-385.
- Shakib K, Norman JT, Fine LG, Brown LR, Godovac-Zimmermann J (2005). Proteomics profiling of nuclear proteins for kidney fibroblasts suggests hypoxia, meiosis, and cancer may meet in the nucleus. *Proteomics*.
- Shen TL, Guan JL (2001). Differential regulation of cell migration and cell cycle progression by FAK complexes with Src, PI3K, Grb7 and Grb2 in focal contacts. *FEBS Lett* 499:176-181.
- Shetty SS, DelGrande D (1994). Inhibition by nickel of endothelin-1-induced tension and associated ⁴⁵Ca movements in rabbit aorta. *J Pharmacol Exp Ther* 271:1223-1227.

- Shevchenko A, Wilm M, Vorm O, Mann M (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* 68:850-858.
- Shi-Wen X, Chen Y, Denton CP, Eastwood M, Renzoni EA, Bou-Gharios G, Pearson JD, Dashwood M, du Bois RM, Black CM, Leask A, Abraham DJ (2004). Endothelin-1 promotes myofibroblast induction through the ETA receptor via a rac/phosphoinositide 3-kinase/Akt-dependent pathway and is essential for the enhanced contractile phenotype of fibrotic fibroblasts. *Mol Biol Cell* 15:2707-2719.
- Shi Y, Brown ED, Walsh CT (1994). Expression of recombinant human casein kinase II and recombinant heat shock protein 90 in Escherichia coli and characterization of their interactions. *Proc Natl Acad Sci U S A* 91:2767-2771.
- Shichiri M, Kato H, Marumo F, Hirata Y (1997). Endothelin-1 as an autocrine/paracrine apoptosis survival factor for endothelial cells. *Hypertension* 30:1198-1203.
- Shichiri M, Sedivy JM, Marumo F, Hirata Y (1998). Endothelin-1 is a potent survival factor for c-Myc-dependent apoptosis. *Mol Endocrinol* 12:172-180.
- Simonson MS, Dunn MJ (1990). Endothelin-1 stimulates contraction of rat glomerular mesangial cells and potentiates beta-adrenergic-mediated cyclic adenosine monophosphate accumulation. *J Clin Invest* 85:790-797.
- Simonson MS, Jones JM, Dunn MJ (1992). Differential regulation of fos and jun gene expression and AP-1 cis-element activity by endothelin isopeptides. Possible implications for mitogenic signaling by endothelin. *J Biol Chem* 267:8643-8649.
- Simonson MS, Wang Y, Herman WH (1996). Nuclear signaling by endothelin-1 requires Src protein-tyrosine kinases. *J Biol Chem* 271:77-82.
- Simpson AW, Stampfl A, Ashley CC (1990). Evidence for receptor-mediated bivalent-entry in A10 vascular smooth-muscle cells. *Biochem J* 267:277-280.

- Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985). Measurement of protein using bicinchoninic acid. *Anal Biochem* 150:76-85.
- Smith SC, Kemp BE, McAdam WJ, Mercer JF, Cotton RG (1984). Two apparent molecular weight forms of human and monkey phenylalanine hydroxylase are due to phosphorylation. *J Biol Chem* 259:11284-11289.
- Sokolovsky M (1992). Structure-function relationships of endothelins, sarafotoxins, and their receptor subtypes. *J Neurochem* 59:809-821.
- Somlyo AP, Somlyo AV (1994). Signal transduction and regulation in smooth muscle. *Nature* 372:231-236.
- Somsel RJ, Wandinger-Ness A (2000). Rab GTPases coordinate endocytosis. *J Cell Sci* 113 Pt 2:183-192.
- Soskic V, Nyakatura E, Roos M, Muller-Esterl W, Godovac-Zimmermann J (1999 a). Correlations in palmitoylation and multiple phosphorylation of rat bradykinin B2 receptor in Chinese hamster ovary cells. *J Biol Chem* 274:8539-8545.
- Soskic V, Gorlach M, Poznanovic S, Boehmer FD, Godovac-Zimmermann J (1999 b). Functional proteomics analysis of signal transduction pathways of the platelet-derived growth factor beta receptor. *Biochemistry* 38:1757-1764.
- Stannard C, Lehenkari P, Godovac-Zimmermann J (2003 a). Functional diversity of endothelin pathways in human lung fibroblasts may be based on structural diversity of the endothelin receptors. *Biochemistry* 42:13909-13918.
- Stannard C, Soskic V, Godovac-Zimmermann J (2003 b). Rapid changes in the phosphoproteome show diverse cellular responses following stimulation of human lung fibroblasts with endothelin-1. *Biochemistry* 42:13919-13928.

Steen H, Kuster B, Fernandez M, Pandey A, Mann M (2002). Tyrosine phosphorylation mapping of the epidermal growth factor receptor signaling pathway. *J Biol Chem* 277:1031-1039.

Stepp DW, Merkus D, Nishikawa Y, Chilian WM (2001). Nitric oxide limits coronary vasoconstriction by a shear stress-dependent mechanism. *Am J Physiol Heart Circ Physiol* 281:H796-H803.

Strehler EE, Zacharias DA (2001). Role of alternative splicing in generating isoform diversity among plasma membrane calcium pumps. *Physiol Rev* 81:21-50.

Strohman R (1994). Epigenesis: the missing beat in biotechnology? *Biotechnology (N Y)* 12:156-164.

Sugawara F, Ninomiya H, Okamoto Y, Miwa S, Mazda O, Katsura Y, Masaki T (1996). Endothelin-1-induced mitogenic responses of Chinese hamster ovary cells expressing human endothelinA: the role of a wortmannin-sensitive signaling pathway. *Mol Pharmacol* 49:447-457.

Suzuki E, Nagata D, Kakoki M, Hayakawa H, Goto A, Omata M, Hirata Y (1999). Molecular mechanisms of endothelin-1-induced cell-cycle progression: involvement of extracellular signal-regulated kinase, protein kinase C, and phosphatidylinositol 3-kinase at distinct points. *Circ Res* 84:611-619.

Takahashi K, Totsune K, Kitamuro T, Sone M, Murakami O, Shibahara S (2002). Three vasoactive peptides, endothelin-1, adrenomedullin and urotensin-II, in human tumour cell lines of different origin: expression and effects on proliferation. *Clin Sci (Lond)* 103 Suppl 48:35S-38S.

Takai Y, Sasaki T, Matozaki T (2001). Small GTP-binding proteins. *Physiol Rev* 81:153-208.

- Takasuka T, Adachi M, Miyamoto C, Furuichi Y, Watanabe T (1992). Characterization of endothelin receptors ETA and ETB expressed in COS cells. *J Biochem (Tokyo)* 112:396-400.
- Takenaka T, Epstein M, Forster H, Landry DW, Iijima K, Goligorsky MS (1992). Attenuation of endothelin effects by a chloride channel inhibitor, indanyloxyacetic acid. *Am J Physiol* 262:F799-F806.
- Takigawa M, Sakurai T, Kasuya Y, Abe Y, Masaki T, Goto K (1995). Molecular identification of guanine-nucleotide-binding regulatory proteins which couple to endothelin receptors. *Eur J Biochem* 228:102-108.
- Takuwa N, Takuwa Y, Yanagisawa M, Yamashita K, Masaki T (1989). A novel vasoactive peptide endothelin stimulates mitogenesis through inositol lipid turnover in Swiss 3T3 fibroblasts. *J Biol Chem* 264:7856-7861.
- Tamura T, Ishihara M, Lamphier MS, Tanaka N, Oishi I, Aizawa S, Matsuyama T, Mak TW, Taki S, Taniguchi T (1995). An IRF-1-dependent pathway of DNA damage-induced apoptosis in mitogen-activated T lymphocytes. *Nature* 376:596-599.
- Tanaka N, Ishihara M, Kitagawa M, Harada H, Kimura T, Matsuyama T, Lamphier MS, Aizawa S, Mak TW, Taniguchi T (1994). Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. *Cell* 77:829-839.
- Taniguchi T, Miyazaki T, Minami Y, Kawahara A, Fujii H, Nakagawa Y, Hatakeyama M, Liu ZJ (1995). IL-2 signaling involves recruitment and activation of multiple protein tyrosine kinases by the IL-2 receptor. *Ann N Y Acad Sci* 766:235-244.
- Teixeira A, Chaverot N, Schroder C, Strosberg AD, Couraud PO, Cazaubon S (1999). Requirement of caveolae microdomains in extracellular signal-regulated kinase and focal adhesion kinase activation induced by endothelin-1 in primary astrocytes. *J Neurochem* 72:120-128.

- Teixeira A, Chaverot N, Strosberg AD, Cazaubon S (2000). Differential regulation of cyclin D1 and D3 expression in the control of astrocyte proliferation induced by endothelin-1. *J Neurochem* 74:1034-1040.
- Terada Y, Yamada T, Takayama M, Nonoguchi H, Sasaki S, Tomita K, Marumo F (1995). Presence and regulation of Raf-1-K (Kinase), MAPK-K, MAP-K, and S6-K in rat nephron segments. *J Am Soc Nephrol* 6:1565-1577.
- Terada Y, Inoshita S, Nakashima O, Yamada T, Tamamori M, Ito H, Sasaki S, Marumo F (1998). Cyclin D1, p16, and retinoblastoma gene regulate mitogenic signaling of endothelin in rat mesangial cells. *Kidney Int* 53:76-83.
- Terrillon S, Barberis C, Bouvier M (2004). Heterodimerization of V1a and V2 vasopressin receptors determines the interaction with beta-arrestin and their trafficking patterns. *Proc Natl Acad Sci U S A* 101:1548-1553.
- Thaw P, Baxter NJ, Hounslow AM, Price C, Waltho JP, Craven CJ (2001). Structure of TCTP reveals unexpected relationship with guanine nucleotide-free chaperones. *Nat Struct Biol* 8:701-704.
- Thomas G (2002). The S6 kinase signaling pathway in the control of development and growth. *Biol Res* 35:305-313.
- Thulasiraman V, Xu Z, Uma S, Gu Y, Chen JJ, Matts RL (1998). Evidence that Hsc70 negatively modulates the activation of the heme-regulated eIF-2alpha kinase in rabbit reticulocyte lysate. *Eur J Biochem* 255:552-562.
- Toker A (1998). Signaling through protein kinase C. *Front Biosci* 3:D1134-D1147.
- Tokito F, Suzuki N, Hosoya M, Matsumoto H, Ohkubo S, Fujino M (1991). Epidermal growth factor (EGF) decreased endothelin-2 (ET-2) production in human renal adenocarcinoma cells. *FEBS Lett* 295:17-21.

- Tokuyama Y, Horn HF, Kawamura K, Tarapore P, Fukasawa K (2001). Specific phosphorylation of nucleophosmin on Thr(199) by cyclin-dependent kinase 2-cyclin E and its role in centrosome duplication. *J Biol Chem* 276:21529-21537.
- Tolias KF, Couvillon AD, Cantley LC, Carpenter CL (1998). Characterization of a Rac1- and RhoGDI-associated lipid kinase signaling complex. *Mol Cell Biol* 18:762-770.
- Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA, Davis RJ (2000). Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science* 288:870-874.
- Touyz RM, Deng LY, Schiffrin EL (1995). Endothelin subtype B receptor-mediated calcium and contractile responses in small arteries of hypertensive rats. *Hypertension* 26:1041-1045.
- Toyo-oka T, Aizawa T, Suzuki N, Hirata Y, Miyauchi T, Shin WS, Yanagisawa M, Masaki T, Sugimoto T (1991). Increased plasma level of endothelin-1 and coronary spasm induction in patients with vasospastic angina pectoris. *Circulation* 83:476-483.
- Turowski P, Myles T, Hemmings BA, Fernandez A, Lamb NJ (1999). Vimentin dephosphorylation by protein phosphatase 2A is modulated by the targeting subunit B55. *Mol Biol Cell* 10:1997-2015.
- Uchide T, Adur J, Fukamachi T, Saida K (2000). Quantitative analysis of endothelin-1 and vasoactive intestinal contractor/endothelin-2 gene expression in rats by real-time reverse transcriptase polymerase chain reaction. *J Cardiovasc Pharmacol* 36:S5-S8.
- Uchide T, Fujimori Y, Sasaki T, Temma K, Adur J, Masuo Y, Kozakai T, Lee YS, Saida K (2002). Expression of endothelin-1 and vasoactive intestinal contractor genes in mouse organs during the perinatal period. *Clin Sci (Lond)* 103 Suppl 48:167S-170S.

- Vacca F, Bagnato A, Catt KJ, Tecce R (2000). Transactivation of the epidermal growth factor receptor in endothelin-1-induced mitogenic signaling in human ovarian carcinoma cells. *Cancer Res* 60:5310-5317.
- van Biesen T, Luttrell LM, Hawes BE, Lefkowitz RJ (1996). Mitogenic signaling via G protein-coupled receptors. *Endocr Rev* 17:698-714.
- van Delft S, Verkleij AJ, Boonstra J, van Bergen en Henegouwen PM (1995). Epidermal growth factor induces serine phosphorylation of actin. *FEBS Lett* 357:251-254.
- Voit R, Hoffmann M, Grummt I (1999). Phosphorylation by G1-specific cdk-cyclin complexes activates the nucleolar transcription factor UBF. *EMBO J* 18:1891-1899.
- Volarevic S, Thomas G (2001). Role of S6 phosphorylation and S6 kinase in cell growth. *Prog Nucleic Acid Res Mol Biol* 65:101-127.
- Wagner-Mann C, Bowman L, Sturek M (1991). Primary action of endothelin on Ca release in bovine coronary artery smooth muscle cells. *Am J Physiol* 260:C763-C770.
- Wandosell F, Serrano L, Avila J (1987). Phosphorylation of alpha-tubulin carboxyl-terminal tyrosine prevents its incorporation into microtubules. *J Biol Chem* 262:8268-8273.
- Wang KK, Wright LC, Machan CL, Allen BG, Conigrave AD, Roufogalis BD (1991). Protein kinase C phosphorylates the carboxyl terminus of the plasma membrane Ca(2+)-ATPase from human erythrocytes. *J Biol Chem* 266:9078-9085.
- Wang L, Gout I, Proud CG (2001). Cross-talk between the ERK and p70 S6 kinase (S6K) signaling pathways. MEK-dependent activation of S6K2 in cardiomyocytes. *J Biol Chem* 276:32670-32677.

- Wang L, Proud CG (2002). Ras/Erk signaling is essential for activation of protein synthesis by Gq protein-coupled receptor agonists in adult cardiomyocytes. *Circ Res* 91:821-829.
- Wang X, Tokuda H, Hirade K, Kozawa O (2002). Stress-activated protein kinase/c-Jun N-terminal kinase (JNK) plays a part in endothelin-1-induced vascular endothelial growth factor synthesis in osteoblasts. *J Cell Biochem* 87:417-423.
- Warner TD, Allcock GH, Corder R, Vane JR (1993). Use of the endothelin antagonists BQ-123 and PD 142893 to reveal three endothelin receptors mediating smooth muscle contraction and the release of EDRF. *Br J Pharmacol* 110:777-782.
- Watschinger B, Sayegh MH (1996). Endothelin in organ transplantation. *Am J Kidney Dis* 27:151-161.
- Webb DJ, Monge JC, Rabelink TJ, Yanagisawa M (1998). Endothelin: new discoveries and rapid progress in the clinic. *Trends Pharmacol Sci* 19:5-8.
- Wegner M (1999). From head to toes: the multiple facets of Sox proteins. *Nucleic Acids Res* 27:1409-1420.
- Wei CM, Lerman A, Rodeheffer RJ, McGregor CG, Brandt RR, Wright S, Heublein DM, Kao PC, Edwards WD, Burnett JC, Jr. (1994). Endothelin in human congestive heart failure. *Circulation* 89:1580-1586.
- Widmann C, Gibson S, Jarpe MB, Johnson GL (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev* 79:143-180.
- Witzmann FA, Jarnot BM, Parker DN, Clack JW (1994). Modification of hepatic immunoglobulin heavy chain binding protein (BiP/Grp78) following exposure to structurally diverse peroxisome proliferators. *Fundam Appl Toxicol* 23:1-8.

- Wong-Dusting HK, Reid JJ, Rand MJ (1989). Paradoxical effects of endothelin on cardiovascular noradrenergic neurotransmission. *Clin Exp Pharmacol Physiol* 16:229-233.
- Woods M, Wood EG, Bardswell SC, Bishop-Bailey D, Barker S, Wort SJ, Mitchell JA, Warner TD (2003). Role for nuclear factor-kappaB and signal transducer and activator of transcription 1/interferon regulatory factor-1 in cytokine-induced endothelin-1 release in human vascular smooth muscle cells. *Mol Pharmacol* 64:923-931.
- Xu D, Emoto N, Giaid A, Slaughter C, Kaw S, deWit D, Yanagisawa M (1994). ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell* 78:473-485.
- Yamauchi J, Miyamoto Y, Kokubu H, Nishii H, Okamoto M, Sugawara Y, Hirasawa A, Tsujimoto G, Itoh H (2002). Endothelin suppresses cell migration via the JNK signaling pathway in a manner dependent upon Src kinase, Rac1, and Cdc42. *FEBS Lett* 527:284-288.
- Yamazaki T, Komuro I, Shiojima I, Yazaki Y (1999). The molecular mechanism of cardiac hypertrophy and failure. *Ann N Y Acad Sci* 874:38-48.
- Yanagida M, Miura Y, Yagasaki K, Taoka M, Isobe T, Takahashi N (2000). Matrix assisted laser desorption/ionization-time of flight-mass spectrometry analysis of proteins detected by anti-phosphotyrosine antibody on two-dimensional-gels of fibroblast cell lysates after tumor necrosis factor-alpha stimulation. *Electrophoresis* 21:1890-1898.
- Yanagisawa H, Hammer RE, Richardson JA, Williams SC, Clouthier DE, Yanagisawa M (1998). Role of Endothelin-1/Endothelin-A receptor-mediated signaling pathway in the aortic arch patterning in mice. *J Clin Invest* 102:22-33.

- Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332:411-415.
- Yanagisawa M (1994). The endothelin system. A new target for therapeutic intervention. *Circulation* 89:1320-1322.
- Yang Z, Krasnici N, Luscher TF (1999). Endothelin-1 potentiates human smooth muscle cell growth to PDGF: effects of ETA and ETB receptor blockade. *Circulation* 100:5-8.
- Yasui Y, Urano T, Kawajiri A, Nagata K, Tatsuka M, Saya H, Furukawa K, Takahashi T, Izawa I, Inagaki M (2004). Autophosphorylation of a newly identified site of Aurora-B is indispensable for cytokinesis. *J Biol Chem* 279:12997-13003.
- Yee HF, Jr., Melton AC, Tran BN (2001). RhoA/rho-associated kinase mediates fibroblast contractile force generation. *Biochem Biophys Res Commun* 280:1340-1345.
- Yeh YC, Burns ER, Yeh J, Yeh HW (1991). Synergistic effects of endothelin-1 (ET-1) and transforming growth factor alpha (TGF-alpha) or epidermal growth factor (EGF) on DNA replication and G1 to S phase transition. *Biosci Rep* 11:171-180.
- Yokomori H, Oda M, Ogi M, Yoshimura K, Nomura M, Fujimaki K, Kamegaya Y, Tsukada N, Ishii H (2003). Endothelin-1 suppresses plasma membrane Ca⁺⁺-ATPase, concomitant with contraction of hepatic sinusoidal endothelial fenestrae. *Am J Pathol* 162:557-566.
- Yoneyama T, Hori M, Tanaka T, Matsuda Y, Karaki H (1995). Endothelin ETA and ETB receptors facilitating parasympathetic neurotransmission in the rabbit trachea. *J Pharmacol Exp Ther* 275:1084-1089.

- Yumet G, Chin MH, Carey B, Soprano KJ, Lipson KE (1995). Endothelin-1 induces gene expression through stimulation of endothelin type A receptors in normal rat kidney cells. *J Cell Physiol* 164:491-498.
- Zachary I, Sinnett-Smith J, Rozengurt E (1992). Bombesin, vasopressin, and endothelin stimulation of tyrosine phosphorylation in Swiss 3T3 cells. Identification of a novel tyrosine kinase as a major substrate. *J Biol Chem* 267:19031-19034.
- Zachary I, Sinnett-Smith J, Turner CE, Rozengurt E (1993). Bombesin, vasopressin, and endothelin rapidly stimulate tyrosine phosphorylation of the focal adhesion-associated protein paxillin in Swiss 3T3 cells. *J Biol Chem* 268:22060-22065.
- Zalcman G, Closson V, Camonis J, Honore N, Rousseau-Merck MF, Tavitian A, Olofsson B (1996). RhoGDI-3 is a new GDP dissociation inhibitor (GDI). Identification of a non-cytosolic GDI protein interacting with the small GTP-binding proteins RhoB and RhoG. *J Biol Chem* 271:30366-30374.
- Zhou Z, Licklider LJ, Gygi SP, Reed R (2002). Comprehensive proteomic analysis of the human spliceosome. *Nature* 419:182-185.
- Zhu W, Zou Y, Shiojima I, Kudoh S, Aikawa R, Hayashi D, Mizukami M, Toko H, Shibasaki F, Yazaki Y, Nagai R, Komuro I (2000). Ca²⁺/calmodulin-dependent kinase II and calcineurin play critical roles in endothelin-1-induced cardiomyocyte hypertrophy. *J Biol Chem* 275:15239-15245.
- Zor T, Selinger Z (1996). Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Anal Biochem* 236:302-308.